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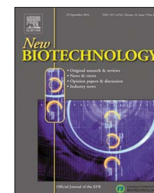

Full length Article

# Heterologous expression of *phaC2* gene and poly-3-hydroxyalkanoate production by recombinant *Cupriavidus necator* strains using canola oil as carbon source

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ABSTRACT

Many heterologous transformation studies have been carried out using the *Cupriavidus necator* $PHB^{-4}$ strain to investigate the expression characteristics of various polyhydroxyalkanoate (PHA) synthase enzymes. In this study, we generated a recombinant *C. necator* $PHB^{-4}$ strain by transforming a plasmid (pMRC03) harbouring the synthetic *phaC2* gene of *Pseudomonas putida* CA-3. Under conditions favourable for expression of the $phaC2_{P.put\ CA\text{-}3}$ gene, canola oil was used as carbon source for the synthesis of PHAs. The expressed synthase polymerised monomers of 3-hydroxybutyrate (3-HB), 3-hydroxyvalerate (3-HV) and 3-hydroxyhexanoate (3-HHx) in the recombinant *C. necator* $PHB^{-4}$ (pMRC03) strain. We then co-expressed the $phaC2_{P.put\ CA\text{-}3}$ gene with the native $phaC1_{C.ne}$ gene in wild type *Cupriavidus necator* H16 (*C. necator* H16 (pMRC03)). This co-expression produced a PHA blend of 3-HB, 3-HV, 3-HHx and 3-hydroxyoctanoate (3-HO) monomers in the presence of canola oil. Gas chromatography analysis revealed the presence of 94 mol% 3-HB, 1 mol% 3-HV, 4 mol% 3-HHx and 1 mol% 3-HO in a tetra-polymer. Thus, we confirmed that a synthetic *phaC2* gene encoding the synthase enzyme is functionally active with substrates ranging from short to medium chain length PHAs.

## Introduction

Polyhydroxyalkanoates (PHAs) are potential substitutes for some petrochemical plastics and materials used in environmental, food, pharmaceutical and biomedical industries [1]. PHAs are synthesised from renewable biomass resources and can be biodegraded to $CO_2$ and $H_2O$ by microbes [2]. PHAs are polyesters formed as linear polymers of 3-hydroxy fatty acids (3HFA). They are classified into short chain length (scl) PHAs formed from monomers of C3–C5 hydroxyacids (HA) and medium chain length (mcl) PHAs formed from monomers of C6–C16 HA [3]. The monomeric composition determines the properties of these polymers, which extend from thermoplastic to elastomeric, with various degrees of crystallinity and adhesive properties [4].

These biopolymers are synthesised primarily by a number of microorganisms as reserve energy sources when nutritional requirements are not balanced [5–7] although some species do not require an imbalance of nutrients to produce PHAs in the early exponential, phase of growth [7]. Extensively studied native PHA producers include *Cupriavidus necator*, *Pseudomonas putida*, *Bacillus subtilis* and *Halomonas* species [3,8–11]. Among these, *C. necator* has the capability to accumulate high amounts of PHA, mainly scl-PHAs, from inexpensive carbon source ranging from simple sugars to complex oil sources [12]. The 3-HA monomers used for PHA synthesis depend on the carbon source and involve different pathways, including those for fatty acid oxidation and biosynthesis pathways and amino acid biosynthesis [13]. The key enzyme, PHA synthase (PhaC), polymerises the 3-HA monomers to produce intracellular polymeric inclusions [5,6].

Regardless of the substrates used, the substrate specificity of different PHA synthases determines the composition of the accumulated PHA polymer [12]. In particular, members of the *Pseudomonas* genus contain synthases with broad substrate specificities that range from scl-PHAs to mcl-PHAs, whereas these synthases from *C. necator* polymerise

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mostly scl-PHAs [14]. At present, two PHA synthase genes, *phaC1* and *phaC2,* have been identified in various *Pseudomonas* spp. (e.g. *P. putida, P. oleovorans*, *P. mendocina* and *P. corrugata*), as well as in other species [15–19]. These *phaC1* and *phaC2* synthase genes are often separated by the *phaZ* gene and are located in a biosynthetic operon, which comprises the six genes involved in PHA synthesis [19,20]. The PhaC1 and PhaC2 proteins have already been characterised for: (1) their expression conditions and levels in the native organisms, (2) the effects of varying substrate specificity based on the available sources, and (3) their monomeric units and amounts of polymer accumulated in the cells. For example, PhaC1 and PhaC2 of *P. stuzeri* 1317 exhibit different substrate specificities [17], whereas their counterparts from *P. mendocina* NK-01 show similar substrate specificity [15]. Previous studies on *P. aeruginosa* have shown that the *phaC1* and *phaC2* genes are not co-transcribed and are regulated independently. In addition, the expression rates of both enzymes vary with respect to the available carbon sources (i.e. glucose and oleic acids) [18,21], although an enzymatic interaction between PhaC1 and PhaC2 is considered highly likely in *Pseudomonas putida* U [5]. The PHA polymers obtained from these two PHA synthase enzymes also display different monomer compositions, molecular weights and physical properties [15–20].

García B et al. [7] described the presence of aromatic biopolyesters synthesised by *Pseudomonas* U, and they used aromatic compounds as precursors to document the organisation of biosynthetic enzymes involved in this biopolymer production. Later, O'Leary et al. [23] examined the synthesis of PHAs with aromatic substituents by *P. putida* CA-3 using styrene as a carbon source. They detected the expression of the *phaC1, phaC2* and *phaZ* genes using three different culture conditions (non-limiting, nitrogen-limiting and carbon-limiting) with styrene as the only carbon source. The *phaC1* gene was expressed constitutively at all limiting conditions, whereas no *phaC2* gene expression was observed under any of the studied conditions. Later, studies using the *P. putida* CA-3 strain confirmed that only PhaC1 synthase plays a vital role in PHA polymer synthesis [24]. This active synthase polymerised a wide range of both aliphatic and aromatic mcl-PHA monomers [23]. The findings from these studies indicated that the functional activity of the PhaC2 synthase of this strain was unknown and needed further investigation, especially since the non-expressed PhaC2 synthase of unknown function exhibited 54% similarity with the active PhaC1 synthase. Table 1 shows a comparison of some PhaC2 synthases of several *Pseudomonas* spp.

Traditionally, enzymes of unknown function are characterised by heterologous expression of their respective genes in host organisms devoid of PHAs. Some examples of these hosts in the present case are *C. necator* PHB$^{-4}$ and *Escherichia coli* [25]. The present study represents the first exploration of the functional activity of the *phaC2* synthase gene product of *P. putida* CA-3 in two host organisms, *C. necator* H16 and *C. necator* PHB$^{-4}$. The versatility in PHA production of these hosts has been known for decades, and they are recognised as having sufficient cytoplasmic cellular space to accumulate as many PHA granules as possible, as well as a stable system for PHA production [26]. An expression plasmid pMRC03 harbouring the synthase gene was constructed and transformed into both host organisms, followed by fermentations by the obtained recombinant strains using canola oil as a non-expensive carbon source for PHA production. Thermo-chemical characterisation analyses confirmed the production of a co-polymer in these recombinant strains that showed heterologous expression of the synthase gene. Taken together, these findings indicate that the PhaC2 synthase functions in the synthesis of PHAs and is capable of incorporating monomers ranging from scl-PHAs to mcl-PHAs.

**Table 1**
Comparison of the *Pseudomonas putida* CA-3 *phaC2* synthase gene sequence with that of diverse *P. putida* sp. synthases.

| Strain | % Identity | GenBank Accession | Reference |
|---|---|---|---|
| Pseudomonas *putida KT2440* | 98 | NP747107 | [47] |
| Pseudomonas *putida BIRD* | 97 | WP_014592664.1 | – |
| Pseudomonas *putidaW619* | 97 | WP_012312401.1 | – |
| Pseudomonas *putida KCTC1639* | 91 | AAP37050 | [48] |
| Pseudomonas *putida S16* | 93 | WP_013974536.1 | – |
| Pseudomonas *putida GM84* | 90 | WP_008099972.1 | – |
| *Pseudomonas putida* U[a] | 87 | AF1150670 | [16] |
| *Pseudomonas fluorescens* GK13[a] | 77 | NC_016830 | [42] |
| *Pseudomonas aeruginosa* PAO1[a] | 77 | NC_002516 | [43] |
| *Pseudomonas sp.* 61.3 | 73 | BAA36202 | [49] |
| *Pseudomonas stutzeri*[a] | 72 | AA059384 | [41] |
| *Pseudomonas USM7-7* | 72 | ACM90523 | [50] |
| *Pseudomonas corrugata*[a] | 71 | AAX92630 | [18] |
| *Pseudomonas resinovorans* | 71 | AF129396 | [51] |
| *Pseudomonas mendocina*[a] | 54 | AEB56599 | [15] |

*phaC2* gene of *Pseudomonas putida* CA-3 was used as query for the BLAST analysis.
[a] Functionally characterised *phaC2* genes.

## Materials and methods

### *Bacterial culture conditions, plasmids and genetic techniques*

*E. coli* DH5 α competent cells (Invitrogen) were used for plasmid transformation. Strains for PHA production (i.e. *Cupriavidus necator* H16 and *C. necator* PHB$^{-4}$; obtained from the Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany) were cultured in nutrient rich (NR) broth at 30 °C [4]. *E. coli* DH5 α was grown in Luria broth (LB) and Terrific broth (TB) at 37 °C [27]; both cultures were shaken at 200 rpm. Chloramphenicol (25 μg/ml) and kanamycin (50 μg/ml) were added to the growth media for recombinant selection.

All DNA manipulations were performed according to Sambrook and Russell [28]. Restriction endonucleases, Klenow fragment and T4 DNA ligase were obtained from New England Biolabs (USA). The *phaC2* synthase gene sequence (1689 bp) of *P. putida* CA-3 was obtained from the NBCI database (accession no. AY714618). This gene sequence was optimised based on codon usage (Codon Usage Database) of *C. necator* [29]. The *phaC2* gene was synthesised and purchased in a cloning vector pUC57 (pRC001) from GenScript Corporation. The pBTB3 (broad host range plasmid) and pK18 plasmids were used as a backbone for constructing the pMRC03 expression plasmid.

### *Construction of the broad host pMRC03 expression plasmid*

An outline of plasmid construction is shown in Fig. 1. Plasmid pMRC01: the 1000-bp arabinose gene fragment was eliminated from pBTB-3 Ranger backbone by BamHI digestion, and blunt ended with Klenow. Plasmid pMRC02: the *phaC2* gene fragment was liberated from the pRC001 plasmid with EcoRI-Hind III digests, blunted with Klenow and sub-cloned in the blunt-repaired BamHI site of the pMRC01 plasmid. pMRC03 expression plasmid: the pK18 plasmid, carrying a constitutive Tac expression system (hybrid promoter composed of trp and lac promoter regions), was linearised by HindIII digestion and cloned into the HindIII site of the pMRC02 plasmid to obtain the final construct. This newly generated plasmid is resistant to chloramphenicol and kanamycin. This broad host pMRC03 construct, harbouring the *phaC2* synthase gene, was transformed into *C. necator* H16 and *C. necator* PHB$^{-4}$ strains via electroporation. The selected recombinant strains, harbouring plasmid pMRC03, were named *C. necator* H16 (pMRC03) and *C. necator* PHB$^{-4}$ (pMRC03).

### *Production and isolation of PHAs*

A 50 ml volume of pre-cultures of the each of the recombinant strains (*C. necator* H16 (pMRC03), *C. necator* PHB$^{-4}$ (pMRC03)) and wild type strains (*C. necator* PHB$^{-4}$, *C. necator* H16) was set up in NR medium. After 24 h, cells were harvested and transferred aseptically to a production medium, which contained, per litre: 10 g fructose, 3.14 g $NH_4SO_4$, 5.66 g $NaH_2PO_4 \cdot 12H_2O$, 1.5 g $KH_2PO_4$, 0.2 g $MgSO_4 \cdot 7H_2O$,

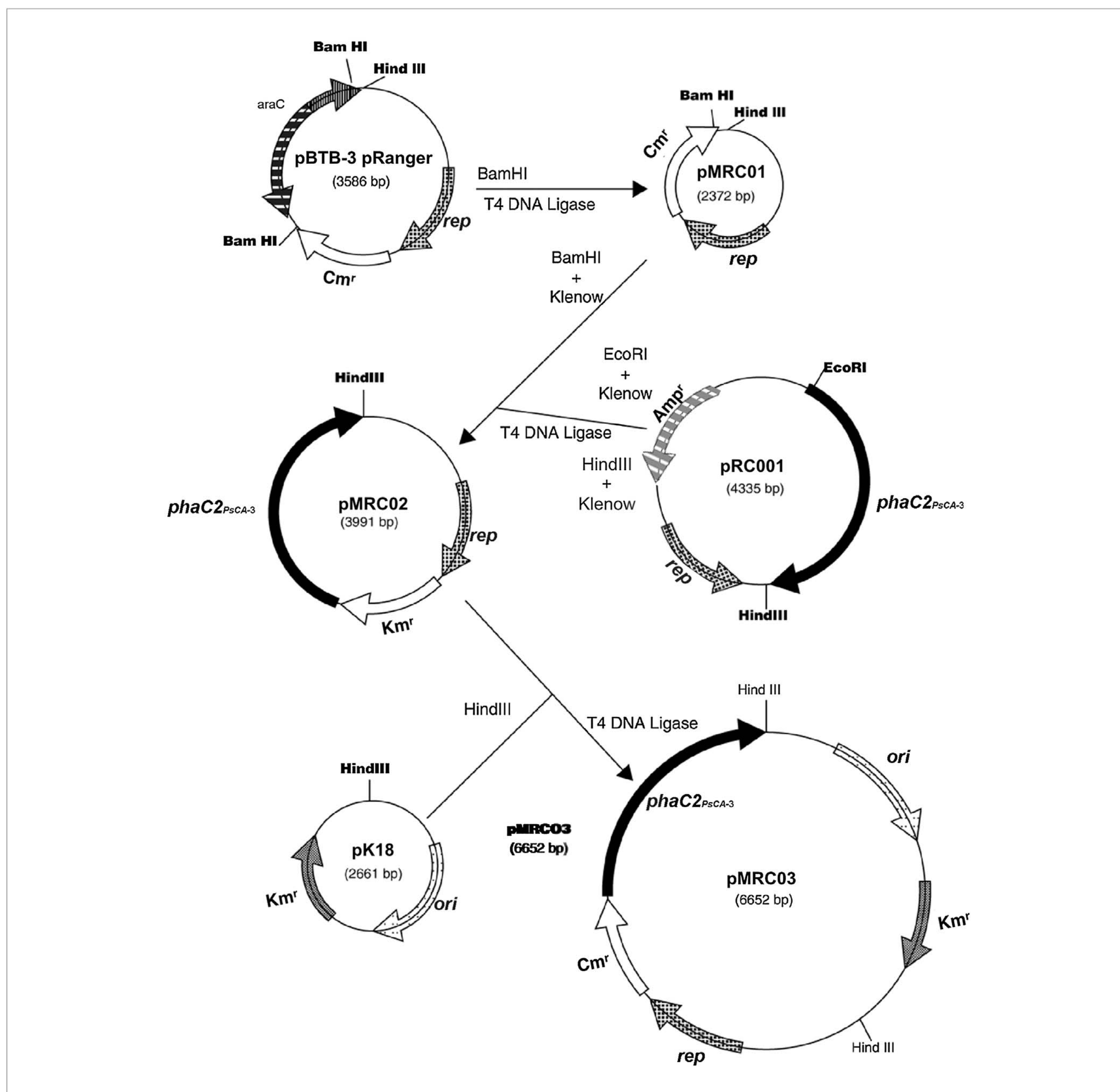


**Fig. 1.** Flowchart for the pMRC03 construct. (Abbreviations: *phaC2*$_{PsCA-3}$: *phaC2* gene, rep: rep protein, Km$^r$: kanamycin resistance, CAT: chloramphenicol acetyl-transferase gene, Cm$^r$: for Chloramphenicol resistance, ori: origin of replication pBBR1).

10 mg $CaCl_2 \cdot 2H_2O$, 20 mg $FeSO_4 \cdot 7H_2O$ and 1 ml microelement solution (0.3 g $H_3BO_3$, 0.2 g $CoCl_2 \cdot 6H_2O$, 0.1 g $ZnSO_4 \cdot 7H_2O$, 30 mg $MnCl_2 \cdot 4H_2O$, 30 mg $Na_2MoO_4 \cdot 2H_2O$, 20 mg $NiCl_2 \cdot 6H_2O$ and 10 mg $CuSO_4 \cdot 5H_2O$, in HCl 0.1N). Chloramphenicol and kanamycin were added when required. At the first stage, 50 ml of production medium was inoculated with a 2.5 ml aliquot of the pre-culture. In the second stage, when the fructose was consumed after 18 h incubation, 5% v/v canola oil was added for the PHA production phase.

The intracellular PHA polyesters were extracted from the dried cell mass in hot chloroform (for 10 min) and precipitated with ten-fold excess ice-cold hexane (this process was repeated twice). The residual solvent was eliminated by evaporation. The PHA contents and compositions from the dried samples were determined by propanolysis. This reaction converts PHA monomers into their related propyl esters. The concentrations of propyl esters were then determined by gas chromatography with an Agilent 6850 gas chromatograph (GC) (Santa Clara, CA) equipped with a DB-Wax column (30 m × 0.32 mm × 0.5 µm; Agilent) and a flame ionisation detector.

*Analytical procedures*

*Determination of thermal properties of PHAs*

Melting temperature ($T_m$), transition temperature ($T_g$) and melting enthalpy ($\Delta H_m$) were determined by differential scanning calorimetry (DSC) with a calorimeter (Perkin-Elmer, USA) equipped with liquid nitrogen cooling accessory. Melt-quenched biopolymer samples (ca. 4 mg) were encapsulated in aluminium pans, cooled at −20 °C and warmed to 200 °C to erase the thermal history. They were subsequently cooled down rapidly to −20 °C, maintained isothermally for 5 min and slowly warmed up to 180 °C with a warming ramp of 5 °C/min. The

degree of crystallinity was determined from the melting enthalpies [30,31]. Polyhydroxybutyrate (PHB) (Goodfellow Cambridge Limited) was used as point of comparison, taking the melting enthalpy of 100% crystalline PHB as a reference.

*Chemical structure analysis of PHAs*

The NMR analyses were done using Bruker equipment (DMX 500 MHz, Germany) and deuterated chloroform as a solvent. The $^{1}$H spectra were obtained at 125 MHz and were analysed using the Spinworks program version 2.5.5.

## Results

*Fermenter culture and functional expression of PhaC2 in recombinant strains*

The inactive PhaC2 of *P. putida* CA-3 was characterised by constructing a pMRC03 expression plasmid containing the *phaC2* gene, as indicated in Fig. 1. The two recombinants strains, *C. necator* PHB$^{-4}$ (pMRC03) and *C. necator* H16 (pMRC03), were then obtained. The functional activity of the PhaC2 enzyme for accumulation of PHAs was analysed and its substrate specificity for 3-HACoA was documented by establishing a three-step fermentation strategy [32] using the recombinant strains. The *C. necator* PHB$^{-4}$ strain was used as a control in this study. The first and second stage growth conditions consisted of a balanced mineral medium with fructose as the carbon source and ammonium sulphate as the nitrogen source (Fig. 2). In the third stage, upon detection of nitrogen limitation, the scl-mcl PHA production phase was initiated by adding canola oil as the carbon source. The *phaC2* gene, under the control of the pTac promoter, was expressed constitutively in all recombinant strains, and the basal expression levels were measured in the recombinant *C. necator* PHB$^{-4}$ (pMRC03) strain. The accumulated polymer was isolated using chloroform and subjected to NMR analysis for chemical composition, as described above.

In the presence of canola oil, the recombinant *C. necator* PHB$^{-4}$ (pMRC03) strain produced a copolymer with monomeric units of 3-hydroxybutyrate (3-HB), 3-hydroxyvalerate (3-HV) and 3-hydroxyhexanoate (3-HHx) (Fig. 3a). A polymer of 3-HB, 3-HV, 3-HHx and 3-hydroxyoctanoate (3-HO) monomers was produced by *C. necator* H16 (pMRC03) (Fig. 3b). Ethyl proton resonance of $CH_2$ group at 1.7 ppm indicated the production of 3-HV and 3-HHx monomer chains by *C. necator* PHB$^{-4}$ (pMRC03). In addition, methine proton signals (CH) appeared at 5.2 ppm [9,33] and methyl proton resonance at 0.89 ppm was assigned to the $CH_3$ groups of 3-HV, 3-HHx and 3-HO monomers [33] produced by *C. necator* H16 (pMRC03). The $CH_2$ group of the HHx and HO monomers was evident at 1.7 ppm.

The relative PHA yields and the repeat-unit compositions of PHA produced by the recombinant *C. necator* H16 strains and the control strain *C. necator* PHB$^{-4}$ are shown in Table 2. The copolymer synthesised by the recombinant *C. necator* PHB$^{-4}$ (pMRC03) had a 3-HB content of up to 96.5 mol%, with significantly enhanced 3-HHx monomer compositions of up to 2.7 mol%; however, only 0.8 mol% of the 3-HV monomer composition was observed. This copolymer estimate was compared with that produced by the wild strain, where 97.9 mol% was 3-HB. The *C. necator* H16 (pMRC03) strain showed 3-HB (94 mol %), 3-HV (1 mol%), 3-HHx (4 mol%) and 3-HO (1 mol%) as the major 3-hydroxyalkanoate repeats units of the polymer after 30 h of cultivation, whereas only 1.4 mol% of mcl 3-HA was estimated in the wild strain. Therefore, it is observable that the incorporation of mcl-PHA monomers is enhanced in the obtained polymer of recombinant strain *C. necator* H16 (pMRC03).

*Thermal characterisation*

The monomeric composition of the polymer is well known to have a

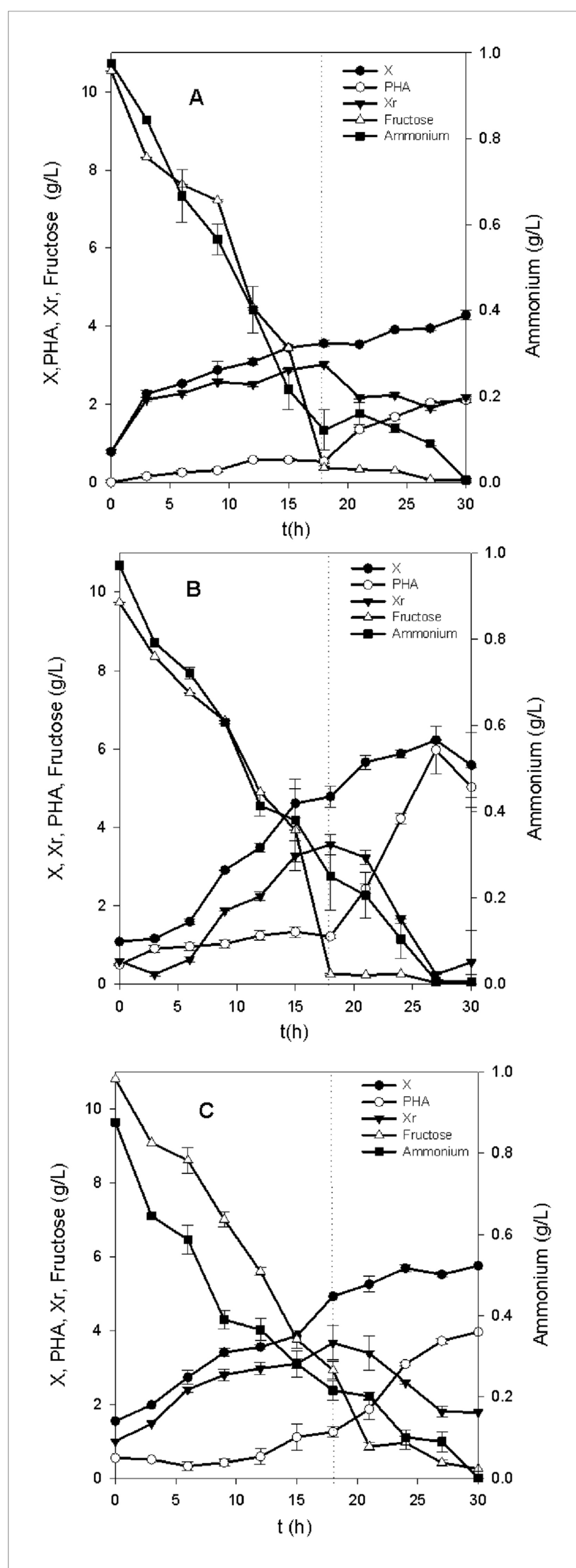


**Fig. 2.** Kinetics of polyhydroxyalkanoate (PHA) production in two stages. A) *C. necator* PHB$^{-4}$ (pMRC03), B) *C. necator* H16 (pMRC03), C) *C. necator* H16. Dotted line indicates the time of canola oil addition. Bars indicate means ± SD of triplicate measurements.

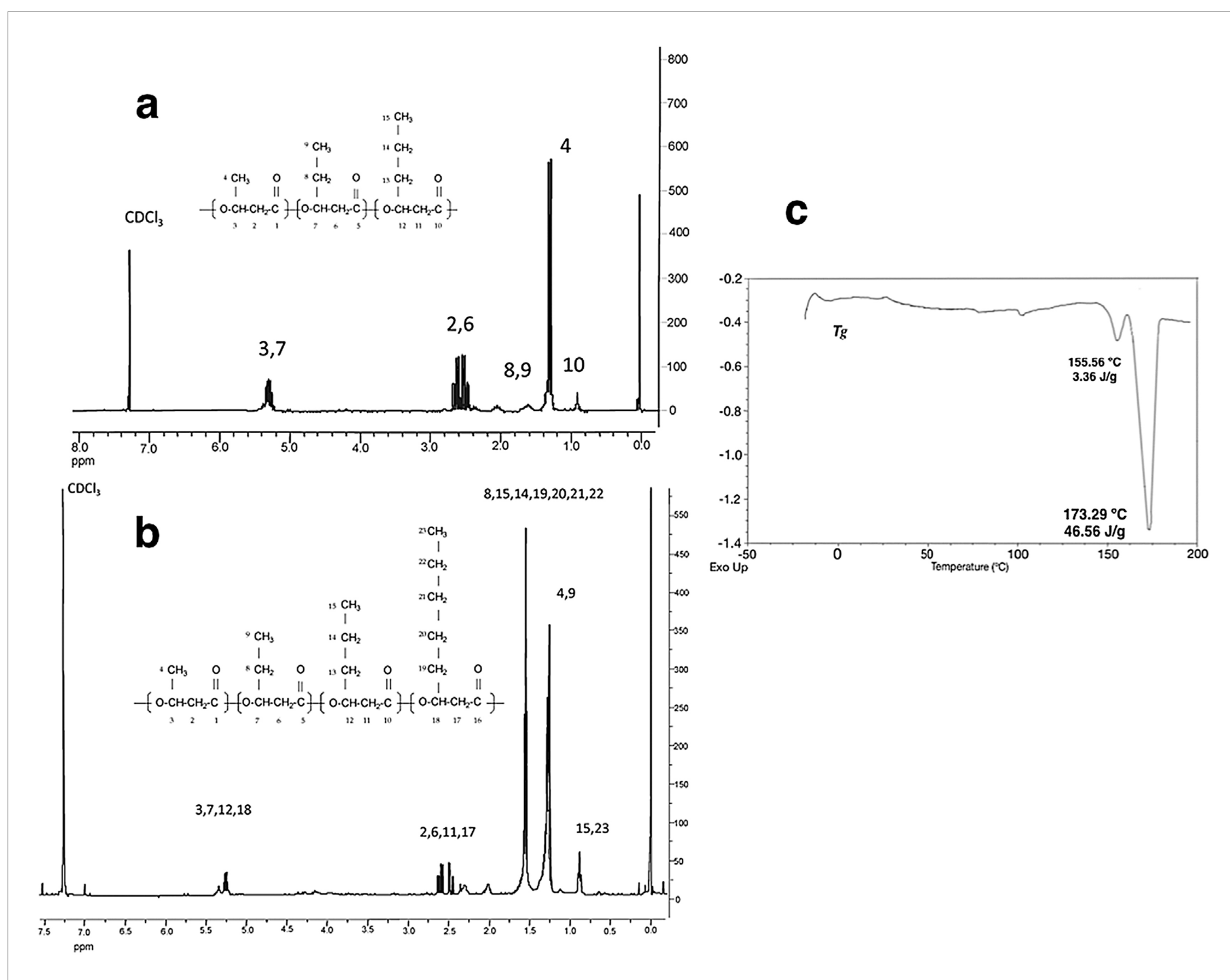


**Fig. 3.** (a) $^1$H NMR spectrum at 500 MHz showing the presence of 3-hydroxybutyrate (3-HB), 3-hydroxyvalerate (3-HV) and 3-hydroxyhexanoate (3-HHx) in the monomers synthesised by *Cupriavidus necator* PHB$^{-4}$ (pMRC03) from canola oil, (b) $^1$H NMR spectrum at 500 MHz showing the presence of 3-HB, 3-HV, 3-HHx, and 3-hydroxyoctanoate (3-HO) in the monomers synthesised by *C. necator* H16 (pMRC03) from canola oil, (c) DSC thermogram of PHAs produced by *C. necator* H16 (pMRC03).

**Table 2**
PHA accumulation by *C. necator* H16 and recombinant strains using canola oil as source.[a]

| Strain | X (CDW)[b] | PHA | PHA composition (mol%) | | | |
|---|---|---|---|---|---|---|
| | (g/l) | (%) | 3-HB[c] | 3-HV[b] | 3-HHx[d] | 3-HO[f] |
| *C. necator* PHB$^{-4}$ (pMRC03) | 3.7 ± 0.3 | 49.0 ± 2.0 | 96.5 | 0.8 | 2.7 | *nd* |
| *C. necator* H16 (pMRC03) | 6.2 ± 0.2 | 96.0 ± 2.0 | 94.0 | 1.0 | 4.0 | 1.0 |
| *C. necator* H16 | 5.7 ± 0.3 | 67.0 ± 0.5 | 97.9 | 0.7 | 1.4 | *nd* |

[a]Incubated for 30 h at 30 °C, initial pH 7.0, 200 rpm. Data shown are means of triplicates.
[b]CDW: cell dry weight.
[c]3-HB: 3-hydroxybutyrate.
[d]3-HV: 3-hydroxyvalerate.
[e]3-HHx: 3-hydroxyhexanoat.
[f]3HHO: 3-Hydroxyoctanoate.
[g]*nd*: not detected.

strong influence on the thermal properties, and this is reflected in the DSC thermograms. The analysis obtained from the thermograms is summarised in Table 3. The $T_m$ of the copolymer produced by *C. necator* PHB$^{-4}$ (pMRC03) was 170 °C. As shown in Fig. 3c and Table 3, two melting points at 155 and 173 °C are observed for the polymer synthesised by the *C. necator* H16 (pMRC03) strain. The enthalpy of fusion was 3.3 J/g at the lowest temperature melting point, whereas for the second point was 47 J/g. These changes in the thermal properties support the incorporation of 3-$HH_X$ and 3-HO mcl monomeric units into the PHA polymer synthesised by *C. necator* H16 (pMRC03). These changes in polymer composition affected the $T_g$ and $T_m$ values, which increased in the PHA blend with a high PHB content [9–34]. Different explanations have been proposed for the presence of multiple melting peaks, including the presence of different molecular weight species, melting-recrystallisation and re-melting processes, and polymorphism [35]. The obtained $T_m$ could indicate that the polymer synthesised by *C. necator* H16 (pMRC03) contains medium chain copolymers. The thermal properties of biopolymers will exhibit differences that agree with their monomeric composition [9]. For example, Sudesh et al. [36] reported melting temperatures fluctuating from 170 to 137 °C depending on the percentage of 3-hydroxyvalerate contained in the heteropolymer chain of PHBV (from 0 to 25%). Even a slight decrease in the melting transition may be attributable to the incorporation of medium-chain monomeric units. For instance, Rathinasabapathy et al. [14] reported a $T_m$ of 176 °C for a polymer that contained small quantities of 3-HV, 3-HHx and 3-HO in a chain composed mainly of hydroxybutyrate units.

**Table 3**
Thermochemical characterization of polyhydroxyalkanoates.

| Strain | Substrate | Sample (Monomeric units composition) | Tg[a](°C) | Tm[c] (°C) | Δ(J/g) | Xc[d](%) | Reference |
|---|---|---|---|---|---|---|---|
| | Fructose | PHB | −2 | 180 | 84.7 | 100 | This study |
| *C. necator* PHB$^{-4}$ (pMRC03) | Canola oil | PHB-*co*-PHV-*co*-PHHx | −5 | 165 | 60 | 70 | This study |
| *C. necator* H16 (pMRC03) | Canola oil | PHB-*co*-PHV-*co*-PHHx-*co*-PHO | −14 | 155–173 | 30 | 35 | This study |
| *C. necator* H16 | Canola oil | PHB-*co*-PHV-*co*-PHHx | −2 | 170 | 78 | 92 | This study |
| *C. necator* H16 | Sunflower oil + sodium valerate | PHB-*co*-PHV | −20 | 170 | 43 | 59 | [4] |
| *C. necator* PHB$^{-4}$-pBBR1MCS-C2 | Crude palm kernel oil + sodium valerate | PHB-*co*-PHV-*co*- PHHx | −4 | 129–144 | 60 | – | [44] |
| *C. necator* PHB$^{-4}$-pBBR1MCS-C2 | Palm kernel oil + Isocaproic acid | PHB-*co*-PHHx | −1 | 129 | 66 | 78 | [45] |

PHB, polyhydroxybutirate; PHV, polyhydroxyvalerate; PHHx,polyhydroxyhexanoate; PHO, polyhydroxyoctanoate.
[a]Glass transition temperature.
[b]Melting temperature.
[c]Enthalpy of fusion.
[d]Cristallynity.

## Discussion

The present study demonstrated the production of PHAs by heterologous expression of a synthetic gene encoding the PhaC2$_{P.put\ CA\text{-}3}$ synthase enzyme in recombinant *C. necator* H16 strains supplied with canola oil as carbon source. The heterologous expression of the PhaC2 synthase enzyme in the non-PHB producing organism resulted in polymer accumulation. The accumulated polymer was purified and further subjected to NMR and DSC characterisations (Fig. 3 and Table 3), which confirmed that the polymer was composed of 3-HB, 3-HV and 3-HHx monomers. Most previous analyses examined the ability of various PhaC2 enzymes from *Pseudomonas* spp. to polymers production [15]. The PhaC2 synthase enzymes (e.g. from *P. mendocina*, and *P. stutzeri* 1317, among others) were able to produce various scl-mcl-PHA copolymers when expressed in recombinant *C. necator* PHB$^{-4}$ strains [37]. In particular, the PhaC2 synthase reported in *P. putida* U was capable of polymerising monomers with lengths of more than five carbon atoms, such as 3-HO and 3-hydroxydecanoate (3-HD), as well as aromatic substrates (e.g. 3-hydroxyphenylvalerate) [16,19,20]. Likewise, the PhaC2 synthase of *P. putida* KCTC1639, which has a very low affinity for mcl-PHA monomers, incorporated only low concentrations of 3-HO and 3-HD monomer units when octanoic acid (C8) and decanoic acid (C10) were used as the carbon sources [38]. By contrast, in the present study, the enzyme showed an affinity primarily for scl-3-hydroxyacyl groups (96.5% 3-HB, 0.8% 3-HV and 2.7% 3-HHx) in *C. necator* PHB$^{-4}$ (pMRC03). Similarly to other PhaC2 synthases, the PhaC2 synthase enzyme from *P. putida* CA-3 also exhibited differences in substrate specificity when compared to the PhaC1 synthase [23]. The PhaC1$_{P.pu\ CA\text{-}3}$ synthase polymerised strictly mcl-PHA monomers, even when the cells were grown in carbon sources such as phenylvaleric acid or butyric acid [39]. Our data indicate that the PhaC2$_{P.puCA\text{-}3}$ synthase enzyme has a broad substrate specificity and is capable of polymerising scl-3HA-CoA and mcl-3HA-CoA carbon monomers as substrates.

By contrast, the PHA concentration in *C. necator* H16 (pMRC03) reached approximately 96% after the addition of canola oil, whereas *C. necator* PHB$^{-4}$ (pMRC03) only synthesised 49% PHA. Both recombinant strains of *C. necator* PHB$^{-4}$ and *C. necator* H16, when supplied with canola oil, accumulated polymers with varying 3-HA monomer fractions in different proportions (Table 2). The notable incorporation of mcl-monomeric units into the polymer was presumably due to the high concentration of monounsaturated fatty acids in canola oil, as these fatty acids are preferentially taken up by *C. necator* [40]. In particular, the formation of 3-HHx and 3-HO monomers probably resulted from the oxidation of oleic acid, which might have been generated by an enoyl-CoA hydratase previously identified in *C. necator* [2]. The incorporation of mcl-PHA monomers was also well supported by the DSC studies. The drop in the melting-transition temperature could be explained by the increase in mcl-monomeric units in the copolymer [35]. For instance, a slight increase in the incorporation of 3-HHx monomers is evident in the *C. necator* PHB$^{-4}$ (pMRC03), when compared with the polymer accumulation in the *C. necator* H16 strain. We observed the production of monomeric units of 3-HV during the fermentation, which raises the question whether the canola oil used in the present study might have contained a low concentration component that was utilised as a substrate for the synthesis of a five-carbon monomer [3,14]. Chen et al. [41] showed a *C. necator* PHB$^{-4}$ strain harbouring a low substrate specificity PHA synthase. After 72 h incubation with pure octanoic acid (an expensive substrate), they obtained a high molar composition of 3-HO monomeric units (52 mol%). In the present study, *C. necator* H16 (pMRC03) accumulated 3-HO monomers in as little as 30 h, using the native PhaC$_{C.ne}$ and PhaC2$_{P.pu\ CA\text{-}3}$ enzymes and metabolising a much less expensive carbon source.

Thus far, studies involving the co-expression of PHA synthase enzymes (with varying substrate specificities) have mostly been conducted using *Pseudomonas* strains, with no reports on the use of *C. necator* for these types of studies. The present study is therefore the first to report on the co-expression of two synthase enzymes (PhaC2 of *P. putida* CA-3 and PhaC1 of *C. necator*) in *C. necator* H16. As mentioned earlier, we heterologously expressed PhaC2$_{P.pu\ CA\text{-}3}$, while PhaC1 $_{C.ne}$ was constitutively expressed in the native organism. The co-expression of these two enzymes resulted in the production of a PHA heteropolymer constructed from 3-HB, 3-HV, 3-HHx and 3-HO monomers (Fig. 3b). Therefore, the incorporation of 3-HO monomers into the heteropolymer produced by *C. necator* H16 (pMRC03) can be attributed to the presence of the PhaC2 enzyme from *P. putida* CA-3. This finding concurs with those of previous co-expression studies on *Pseudomonas* organisms in which the co-expressed PHA synthase enzymes acted independently to produce PHA polymer [15,17,41,46]. Moreover, the co-expression strategy significantly enabled the incorporation of multiple monomers to produce novel PHA polymers. This opens up a broader use of free fatty acids as substrates for PHA production in future investigations.

## Conclusion

A synthetic gene encoding PhaC2 synthase of *P. putida* CA-3 was heterologously expressed in *C. necator* H16 and *C. necator* PHB$^{-4}$ strains. The expressed synthase is functionally active and gives rise to accumulation of PHA polymer. The scl-mcl-3-HA CoA monomers were also incorporated into the obtained polymer when canola oil was supplied as a non-expensive carbon source. This implies that the PhaC2 synthase has a broad substrate specificity. However, the capability for monomer incorporation depended on the availability of the carbon sources and the host. Accordingly, future investigations will focus on the analysis of the substrate specificity of the *P. putida* CA-3 PhaC2 synthase, with the aim of incorporating aromatic constituents into the PHA polymer as occurs with the PhaC1 synthase.

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