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Biosynthesis of P(3HB-*co*-3HV-*co*-3HHp) terpolymer by *Cupriavidus necator* PHB⁻4 transformant harboring the highly active PHA synthase gene of *Chromobacterium* sp. USM2

Devi-Nair Rathi[1], Elin Persson Jutemar[2], Frans H. J. Maurer[2] and Kumar Sudesh[1]*

[1]Ecobiomaterial Research Laboratory, School of Biological Sciences, Universiti Sains Malaysia, 11800 Penang, Malaysia.
[2]Polymer and Materials Chemistry, Department of Chemistry, Lund University, P.O. Box 124, SE-221 00 Lund, Sweden.
Email: ksudesh@usm.my

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ABSTRACT

Aims: This study evaluates potentials of *Cupriavidus necator* PHB⁻4 transformant harboring the highly active polyhydroxyalkanoate synthase gene (*phaC*) of a locally isolated *Chromobacterium* sp. USM2 for its ability to incorporate 3-hydroxyheptanoate (3HHp) monomer.
Methodology and results: A mixture of fructose and sodium heptanoate fed to the culture gave rise to poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-3-hydroxyheptanoate), [P(3HB-*co*-3HV-*co*-3HHp)] terpolymer synthesis, with traces of 3HHp monomers confirmed through gas chromatography (GC), proton (^{1}H) and carbon (^{13}C) NMR spectra.
Conclusion, significance and impact of study: This study has revealed that the PHA synthase of *Chromobacterium* sp. USM2 has a broad range of substrate specificity. The synthase is able to polymerize 3-hydroxyalkanoate monomers having 4–7 carbon atoms.

Keywords: Cupriavidus necator, *Chromobacterium* sp. USM2, sodium heptanoate, P(3HB-*co*-3HV-*co*-3HHp) terpolymer

INTRODUCTION

Plastics have become an essential part of our daily life. The petrochemical-based plastics production is however not sustainable and contributes to the exploitation of landfill areas as well as some environmental pollution issues. The introduction of alternative sustainable resources for the production of plastics is highly desirable and polyhydroxyalkanoates (PHAs), which resemble some common plastic materials, may be able to mitigate these issues. PHAs are synthesized biologically by various microorganisms under certain nutrient limitation (such as nitrogen or phosphorous) with surplus of carbon source (Madison and Huisman, 1999). PHAs have captured much interest due to its fully biodegradable nature as well as sustainable production through consumption of renewable carbon sources (Braunegg *et al.*, 1998; Sudesh and Iwata, 2008).

PHA synthesis via the polymerization of various monomers by a bacterial strain very much depends on the carbon substrate, as well as the substrate specificity of the polymerizing enzyme, PHA synthase. The incorporation of different types and ratio of monomers determines properties of the PHA. Poly(3-hydroxybutyrate), P(3HB) is the most commonly synthesized form of PHA. However, application of this homopolymer is limited as it is highly crystalline, stiff and brittle in nature (Freier, 2006). These polymer properties could be improved through the incorporation of co-monomers such as 3-hydroxyvalerate (3HV) (Steinbüchel *et al.*, 1993), 3-hydroxyhexanoate (3HHx) (Doi *et al.*, 1995), 4-hydroxybutyrate (4HB) (Nakamura *et al.*, 1992) and also the recently discovered 3-hydroxy-4-methylvalerate (3H4MV) (Tanadchangseng *et al.*, 2009; Lau *et al*., 2012).

Among the various types of bacteria that are capable of producing PHAs, *Cupriavidus necator* have gained the most attention because it is capable of utilizing a wide range of carbon substrates (such as sugars and triglycerides) as well as growing to high cell densities. However, the PHA polymerizing ability of this strain is limited to short chain-length (SCL_{PHA}) (Sudesh *et al.*, 2000; Yuan *et al.*, 2001). In order to enable this strain to produce a wider range of PHAs, the PHA-negative mutant, *C. necator* PHB⁻4 has been genetically modified to express heterologous PHA synthase with wider substrate specificity. In addition to genetic modification, the addition of precursor carbon compounds is also necessary to initiate the formation of co-monomers. Some of these precursor compounds are highly toxic and can impair cell growth and PHA accumulation (Park *et al.*, 2001; Bhubalan *et al*., 2008). Thus, utilization of a

*Corresponding author

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particular strain with higher affinity towards a particular monomer, coupled with specific precursor feeding strategies might minimize toxicity effects on the cell metabolism.

For example, *Chromobacterium* sp. USM2 synthase is known to possess high affinity towards 3HV monomer synthesis; which permits efficient synthesis of 3HV with low concentration of precursor (Steinbüchel *et al.*, 1993; Kolibachuk *et al.*, 1999; Bhubalan *et al.*, 2010a; Bhubalan *et al.*, 2010b). *Chromobacterium violaceum* is also known to possess broad substrate specificity which allows polymerization of C_4-C_8 monomers (Kolibachuk *et al.*, 1999). The recently discovered *Chromobacterium* sp. USM2 is also capable of synthesizing PHA containing 3HV monomer as much as 98 mol%, in the presence of suitable precursors (Bhubalan *et al.*, 2010a).

Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)] copolymers can be produced by *C. necator* PHB$^-$4 harboring the *phaC* of *Aeromonas caviae* when fed with hexanoate or octanoate (Fukui and Doi, 1997). Further studies reported on the production of a novel terpolymer of P(3HB-*co*-3HV-*co*-3HHp) by the same strain from alkanoic acids with odd-numbered carbon (Fukui *et al.*, 1997). The expression of *phaC* from *A. caviae* as well as *Chromobacterium* sp. USM2 in *C. necator* PHB$^-$4 were reported to successfully synthesize P(3HB-*co*-3HV-*co*-3HHx) terpolymers from palm oil with the addition of 3HV-precursors; namely propionic and valeric acid (Bhubalan *et al.*, 2008; Bhubalan *et al.*, 2010a; Bhubalan *et al.*, 2010b). The PHA synthase of *Chromobacterium* sp. USM2 has also been shown to polymerize 3H4MV monomer that contains 6 carbon atoms. In this study, we further show that the *Chromobacterium* sp. USM2 synthase has the ability to polymerize 3-hydroxyheptanoate (3HHp) monomer that contains 7 carbon atoms.

MATERIALS AND METHODS

Bacterial strain

PHA-negative mutant, *C. necator* PHB$^-$4 transformant harboring PHA synthase gene of *Chromobacterium* sp. USM2 (PHB$^-$4/pBBR1MCS-C2) was used in this study. Construction of this plasmid has been described in detail elsewhere (Bhubalan *et al.*, 2010a). *C. necator* PHB$^-$4 transformant harboring PHA synthase gene of *A. caviae* (PHB$^-$4/PBBREE32d13) was also evaluated (Tsuge *et al*., 2004).

PHA biosynthesis

PHA biosynthesis was carried out via one-stage cultivation. Pre-culture was prepared by cultivating the cells in 50 mL of nutrient rich (NR) broth at 30° C and 200 rpm. NR broth were comprised of 10 g/L peptone, 10 g/L meat extract and 2 g/L yeast extract (pH 7.0). Approximately 7 h upon cultivation, 3% (v/v) cells (OD_{600} = 4) were inoculated into nitrogen-limiting mineral salts medium (MM). The MM broth consisted of 3.32 g/L Na_2HPO_4, 2.80 g/L KH_2PO_4, 0.50 g/L NH_4Cl, 0.25 g/L $MgSO_4{\cdot}7H_2O$ and 1 mL/L trace elements (Doi *et al.*, 1995). The trace element solution were made up of 0.22 g $CoCl_2{\cdot}6H_2O$, 9.7 g $FeCl_3$, 7.8 g $CaCl_2$, 0.12 g $NiCl_2{\cdot}6H_2O$, 0.11 g $CrCl_3{\cdot}6H_2O$, 0.16 g $CuSO_4{\cdot}5H_2O$ in 1 L 0.1 N HCl (Kahar *et al.*, 2004). Fructose (2%) was used as the carbon source and added during inoculation. Sodium heptanoate (3 g/L) was chosen to initiate the production of both 3HV and 3HHp monomers. Both fructose and sodium heptanoate were autoclaved separately at 121°C for 15 min under 15 psi (103 kPa). Kanamycin (50 µg/mL) was added into both media for plasmid maintenance. Upon predetermined cultivation period, the culture was harvested by centrifugation (4°C, 9370 *g*, 5 min), followed by washing with distilled water and finally lyophilized prior to further analysis.

Analytical procedures

Several analyses were carried out in order to determine the monomer composition of the PHA produced by the transformant.

Gas chromatography (GC) analysis

PHA quantification was carried out via GC analysis using caprylic methyl ester (CME) as the internal standard for determination of PHA content, monomer composition and purity determination of the extracted polymers. Approximately 20 mg of lyophilized cells were weighed into screw-cap test tubes and subjected for methanolysis (140 min, 100 °C) in presence of 85% (v/v) methanol and 15% (v/v) sulphuric acid (Braunegg *et al*., 1978). The resulting methyl esters were then analyzed by GC (*Shimadzu GC-2010 AF 230LV*) equipped with a capillary column SPB-1 (30 m length, 0.25 mm internal diameter, and 0.25 mm film thickness; Supelco, Bellefonte, PA, USA) connected to a flame ionization detector. Nitrogen gas was used as the carrier gas (1 mL/min), and the chloroform-dissolved sample (2 mL) was injected using an auto injector (*Shimadzu AOC-20i*). The injector and detector temperatures were set at 270 and 280 °C, respectively. The column temperature was increased from 70 to 280 °C at 10 °C/ min.

Polymer extraction

PHA was extracted from lyophilized cells by stirring approximately 1 g of cells in 100 mL of chloroform at room temperature for 5 days. The mixture was filtered using *Whatman 3* filter paper, concentrated by rotary evaporator to approximately 15 mL, and then precipitated out in 100 mL of rapidly stirred chilled methanol. The purified PHA was then air-dried and subjected to nuclear magnetic resonance (NMR) analysis.

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NMR analysis

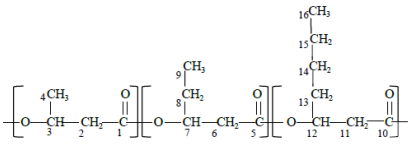
NMR spectroscopy was performed at 25 °C on polymer samples dissolved in deuterated chloroform ($CDCl_3$) using *Bruker AVANCE 400 FT*-nuclear magnetic resonance operating at 400 and 300 MHz for ^{1}H and ^{13}C NMR analysis, respectively. The chemical shifts were reported in ppm in relative to tetramethylsilane (Me_4Si) signals.

RESULTS AND DISCUSSION

The *C. necator* PHB^{-}4 transformant harboring *phaC* of *Chromobacterium* sp. USM2 (*phaC*$_{Cs}$) have been reported to synthesize copolymers and terpolymers of P(3HB-*co*-3HHx) and P(3HB-*co*-3HV-*co*-3HHx) in mixtures of palm oil and 3HV-precursors (Bhubalan *et al.*, 2010b). Similarly, *C. necator* PHB^{-}4 transformant harboring *phaC* of *A. caviae* was also reported to biosynthesize P(3HB-*co*-3HHx) copolymer, as well as P(3HB-*co*-3HV-*co*-3HHx) and P(3HB-*co*-3HV-*co*-3HHp) terpolymers (Fukui and Doi, 1997; Fukui *et al.*, 1997; Bhubalan *et al.*, 2008).

In this study, *C. necator* PHB^{-}4 transformant harboring *phaC* of *Chromobacterium* sp. USM2 (*phaC*$_{Cs}$) and *A. caviae* (*phaC*$_{Ac}$) demonstrated the ability to incorporate 3HV and 3HHp monomer when sodium heptanoate was added as the precursor. This was confirmed by ^{1}H and ^{13}C NMR spectra as presented in Figures 1, 2 and 3; respectively. These spectra's were compared to previous reports on the production of 3HHp monomers, namely ^{1}H (Wang *et al.*, 2009) and ^{13}C (Fukui *et al.*, 1997).

^{1}H NMR spectrum showed the presence of P(3HB-*co*-3HV-*co*-3HHp) terpolymer synthesized by *C. necator* transformant harboring the PHA synthase gene of *Chromobacterium* sp. USM2 (Figure 1). The chemical shifts at 0.90 ppm corresponded to methyl group (-CH_3) of 3HV and 3HHp, while chemical shifts at 1.20 ppm corresponded to methyl group of 3HB. Similarly, the methylene (-CH_2) and methine (-CH) groups of 3HHp monomer were indicated by chemical shifts around 1.20 – 1.30, 1.60–1.70, 2.40–2.60 ppm and 5.10–5.30 ppm, respectively. On the other hand, methylene (-CH_2) groups of 3HV monomer were indicated by chemical shifts around 1.60–1.70, 2.40–2.60; while methine (-CH) groups were identified around 5.10–5.30 ppm. Finally, presence of methylene (-CH_2) and methine (-CH) groups of 3HB monomer were confirmed by chemical shifts around 2.40–2.60 and 5.10–5.30 ppm. This spectrum is also equal to a simple P(3HB-*co*-3HV) copolymer (Figure 1).



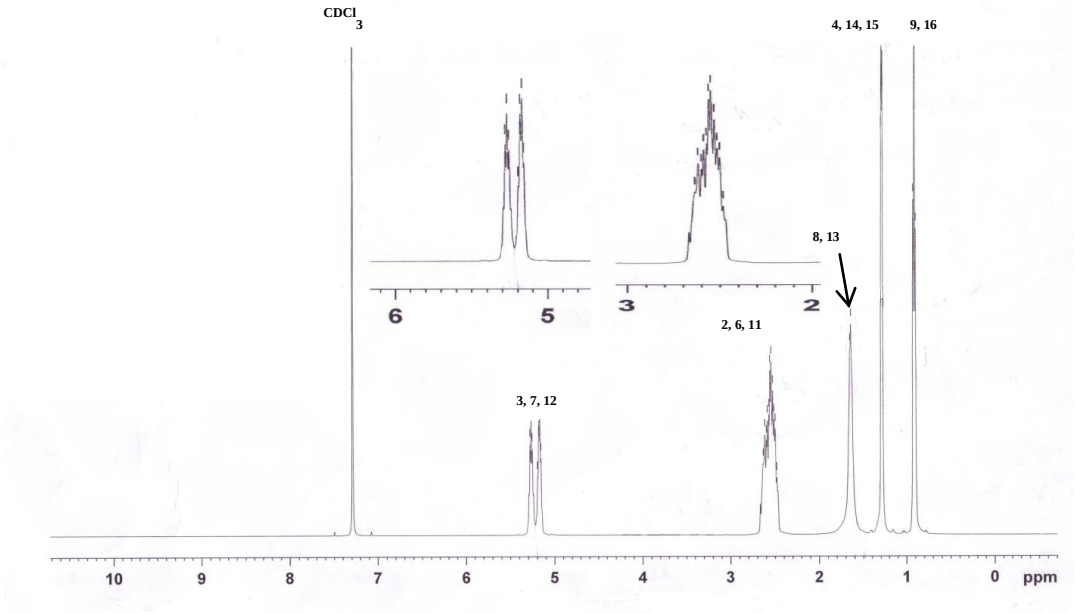


Figure 1: ^{1}H NMR spectrum is consistent with the formation of 3HHp monomer in P(3HB-*co*-3HV-*co*-3HHp) terpolymer produced by *C. necator* PHB^{-}4 harboring *phaC*$_{Cs}$ in mixtures of fructose and sodium heptanoate (24 h culture).

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On the other hand, synthesis of P(3HB-*co*-3HV-*co*-3HHp) terpolymer could be confirmed via ^{13}C NMR. As shown in Figures 2 and 3, chemical shifts around 9.0, 14.0 and 19.0 ppm corresponded to methyl carbon (-CH_3) of 3HV, 3HHp and 3HB monomer, respectively. Methylene carbon (-CH_2) of 3HB, 3HV and 3HHp monomers were indicated by chemical shifts around 22.0–33.0 ppm and 38.0–40.0 ppm. Lastly, chemical shifts around 67.0–70.0 ppm indicated presence of methine (-CH) carbon of all three monomers studied (Figure 2).

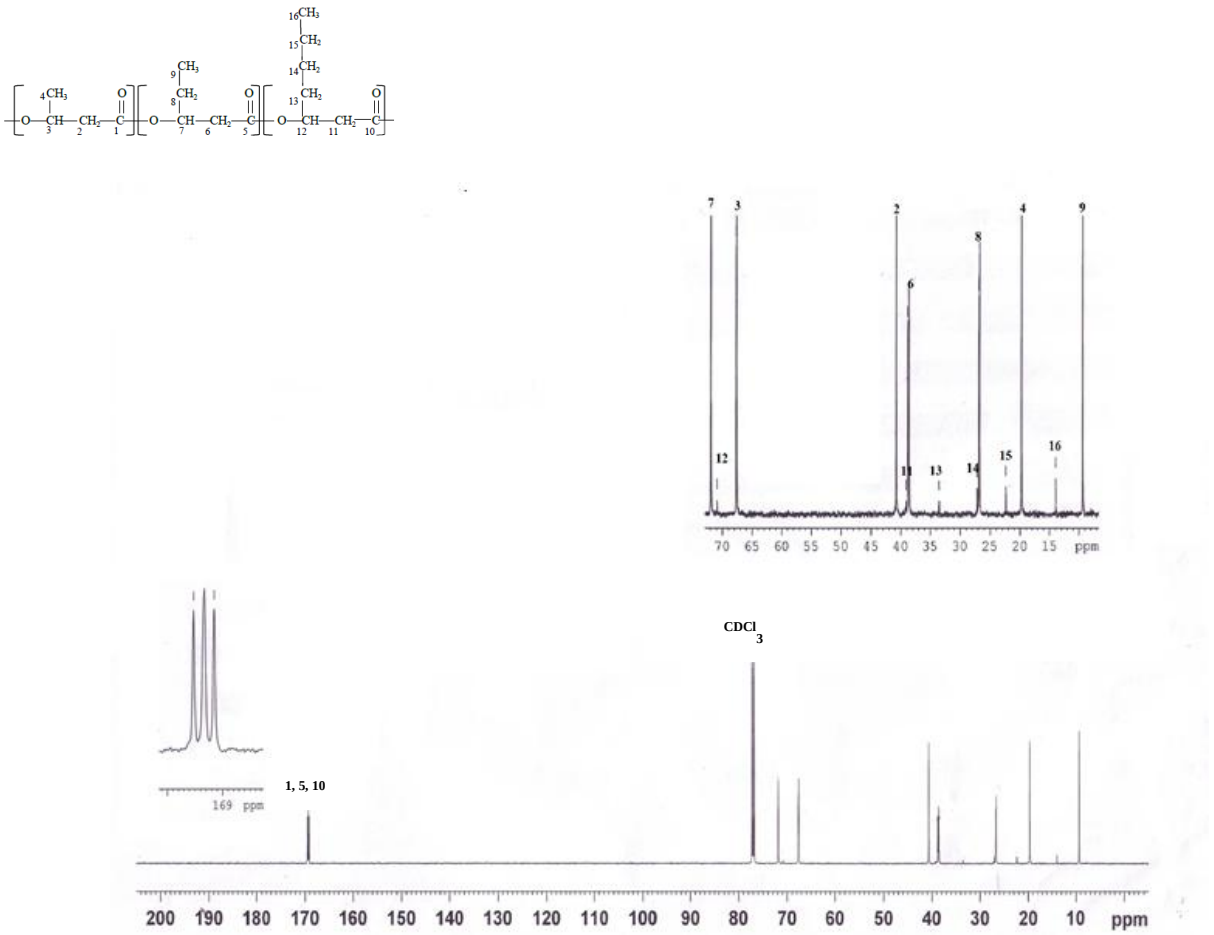

Figure 2: ^{13}C NMR spectrum confirming the presence of 3HHp monomer in P(3HB-*co*-3HV-*co*-3HHp) terpolymer produced by *C. necator* PHB^{-}4 transformant harboring *phaC*$_{Cs}$ in mixtures of fructose and sodium heptanoate (24 h culture).

As presented in Tables 1 and 2, only trace amounts of 3HHp monomers were detected via GC upon cultivation for different duration. In contrast to earlier studies that utilized sodium valerate and sodium propionate for the generation of 3HV monomers, this study demonstrated the ability of the strain to incorporate 3HV monomers from sodium heptanoate. Sodium heptanoate generated synthesis of both 3HV and 3HHp monomers, with more intermediates channeled for 3HV synthesis. *C. necator* PHB^{-}4 transformant harboring *phaC*$_{Cs}$ gave rise to higher incorporation of 3HV monomers in comparison to those of *phaC*$_{Ac}$ (Table 1 and 2).

This phenomenon could be possibly due to higher affinity of *Chromobacterium* sp. synthase towards 3HV monomers. An earlier study conducted by Fukui and co-workers presented similar synthesis of P(3HB-*co*-3HV-*co*-3HHp) terpolymer by *C. necator* PHB^{-}4 transformant harboring *phaC*$_{Ac}$, and hence used as a comparison in this study (Fukui *et al*., 1997). Traces of 3HHp monomers up to 10 mol% was detected from various odd-numbered alkanoic acids such as heptanoate, valerate, nonanoate and others (Fukui *et al*., 1997) Although toxicity effects exerted by sodium heptanoate is higher, yet the ability of the strains to accumulate reasonable amount of 3HV molar fractions can be regarded as an added advantage along with traces of 3HHp monomers.

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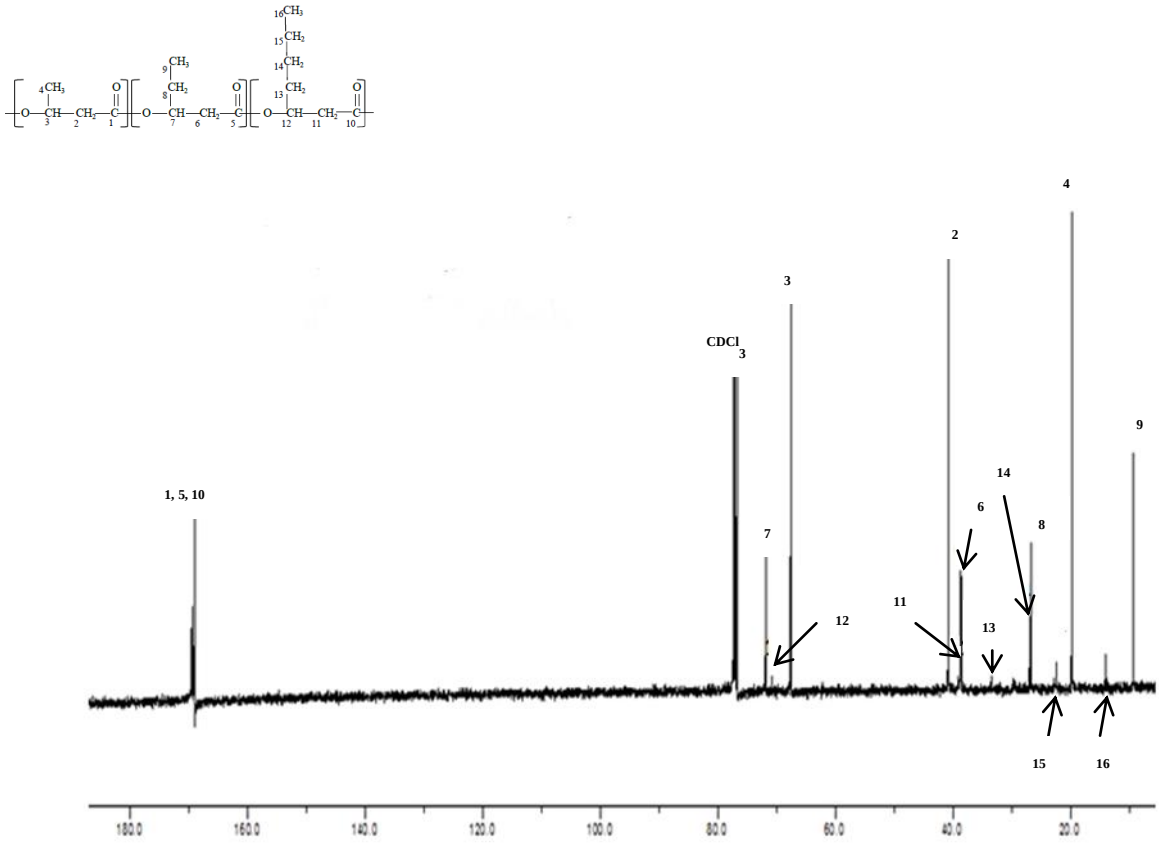

Figure 3: ^{13}C NMR spectrum confirming the presence of 3HHp monomer in P(3HB-*co*-3HV-*co*-3HHp) terpolymer produced by *C. necator* PHB^{-}4 transformant harboring *phaC*$_{Ac}$ in mixtures of fructose and sodium heptanoate (24 h culture).

Table 1: Biosynthesis of P(3HB-*co*-3HV-*co*-3HHp) terpolymer by *C. necator* PHB^{-}4 transformant harboring *phaC*$_{Cs}$ in mixtures of fructose and sodium heptanoate.

Cultivation period (h)[a]	CDW (g/L)	PHA content (wt%)[b]	PHA concentration (g/L)[c]	Residual biomass (g/L)[c]	Monomer composition (mol%)		
					3HB	3HV	3HHp
18	1.0	40 ± 1	0.4	0.6	49	51	Tr*
24	2.1 ± 0.1	44 ± 2	0.9	1.2 ± 0.1	55	45	Tr*

Data shown are by means of triplicate.
[a] Incubated at 30 °C and 200 rpm, in MM supplemented with 2% (w/v) fructose and 3 g/L sodium heptanoate. Carbon source and precursor were added during inoculation (0 h).
[b] PHA content in freeze-dried cells were determined via gas chromatography (GC).
[c] PHA concentration = PHA content × CDW
Residual biomass = CDW – PHA concentration
*Tr = Trace amount

Table 2: Biosynthesis of P(3HB-*co*-3HV-*co*-3HHp) terpolymer by *C. necator* PHB^{-}4 transformant harboring *phaC*$_{Ac}$ in mixtures of fructose and sodium heptanoate.

Cultivation period (h)[a]	CDW (g/L)	PHA content (wt%)[b]	PHA concentration (g/L)[c]	Residual biomass (g/L)[c]	Monomer composition (mol%)		
					3HB	3HV	3HHp
18	1.2 ± 0.1	31 ± 2	0.4	0.8	67	33	Tr*
24	2.0	33 ± 3	0.6 ± 0.1	1.3	77	23	Tr*

Data shown are by means of triplicate.
[a] Incubated at 30 °C and 200 rpm, in MM supplemented with 2% (w/v) fructose and 3 g/L sodium heptanoate. Carbon source and precursor were added during inoculation (0 h).
[b] PHA content in freeze-dried cells were determined via gas chromatography (GC).
[c] PHA concentration = PHA content × CDW
Residual biomass = CDW – PHA concentration
*Tr = Trace amount

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CONCLUSION

This study has revealed that the PHA synthase of *Chromobacterium* sp. USM2 has a broad range of substrate specificity. The synthase is able to polymerize from four-carbon (3HB) monomer up to seven-carbon (3HHp) monomer. In addition to P(3HB-*co*-3HV-*co*-3HHp) terpolymer production, a novel P(3HB-*co*-3HV-*co*-3HHx-co-3HHp) copolymer could probably also be synthesized by using mixtures of crude palm kernel oil (CPKO) and sodium heptanoate.

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