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Cloning and expression of the PHA synthase gene from a locally isolated *Chromobacterium* sp. USM2

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ABSTRACT

Chromobacterium sp. USM2, a locally isolated bacterium was found to synthesize poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate), P(3HB-*co*-3HV) copolymer with high 3HV monomer composition. The PHA synthase gene was cloned and expressed in *Cupriavidus necator* PHB^{-}4 to investigate the possibilities of incorporating other monomer. The recombinant successfully incorporated 3-hydroxyhexanoate (3HHx) monomer when fed with crude palm kernel oil (CPKO) as the sole carbon source. Approximately 63 ± 2 wt% of P(3HB-*co*-3HHx) copolymer with 4 mol% of 3HHx was synthesized from 5 g/L of oil after 48 h of cultivation. In addition, P(3HB-*co*-3HV-*co*-3HHx) terpolymer with 9 mol% 3HV and 4 mol% 3HHx could be synthesized with a mixture of CPKO and sodium valerate. The presence of 3HV and 3HHx monomers in the copolymer and terpolymer was further confirmed with $^{+}$H-NMR analysis. This locally isolated PHA synthase has demonstrated its ability to synthesize P(3HB-*co*-3HHx) copolymer from a readily available and renewable carbon source; CPKO, without the addition of 3HHx precursors.

Keywords: Chromobacterium sp., poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate), poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate), crude palm kernel oil, sodium valerate

INTRODUCTION

Extensive studies have been carried out since the discovery of polyhydroxyalkanoate (PHA) early last century to exploit this intriguing microbial polymer. This naturally occurring carbon and energy storage compound is found in various bacteria and its formation is known to be induced usually by unfavorable growth conditions (Anderson and Dawes, 1990; Doi, 1990). The identification of various hydroxyalkanoate (HA) units besides 3-hydroxybutyrate (3HB) proved to have major impact on the research of this bacterial reserve polymer (Sudesh *et al*., 2000). To date, around 150 different PHA monomers have been reported (Steinbüchel and Lütke-Eversloh, 2003). These monomers are classified into two groups based on the number of carbon atoms in their chemical structure. Those containing 3-5 carbon atoms are deemed as short-chain-length (SCL) PHA while medium-chain-length (MCL) PHA consists of 6-14 carbon atoms (Steinbüchel and Lütke-Eversloh, 2003).

PHA that resembles the main commodity plastics (e.g. polypropylene and polystyrene) can be designed and synthesized to suit numerous applications ranging from stiff packaging goods to highly elastic materials for coating (Sudesh and Iwata, 2008). Due to its thermal stability, PHA can be heat-processed using similar technologies in the current plastic industry with an added advantage of complete biodegradability in the natural environment (Doi, 1990; Sudesh *et al*., 2000). PHAs can be produced from renewable resources such as sugars and plant oils (Loo and Sudesh, 2007a). Attractive properties of PHA make it a suitable substitute for some of the petrochemical-based synthetic plastics. Among the various types of PHA, poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)] has been shown to possess useful properties such as softness and flexibility (Doi *et al*., 1995). In addition, it has also been demonstrated that P(3HB-*co*-3HHx) having low molar fraction of 3HHx can be produced from vegetable oils as the sole carbon source without having to add 3HHx precursors (Fukui and Doi, 1998).

Production of PHA by both Gram negative and Gram positive bacteria have been investigated and well documented. Besides this, isolation and identification of new bacterial strains with better PHA production capabilities are also being continuously investigated. In a recent study, a locally isolated *Cupriavidus* strain from the Malaysian environment had been evaluated for the production of PHA copolymer from various carbon sources (Vigneswari *et al*., 2009). Amongst the documented PHA producing microorganisms, the strain of interest related to this study is *Chromobacterium violaceum.* It was identified as a PHA producer and is known to populate the soil and water in areas of tropical biodiversity around the world (Kolibachuk *et al*., 1999). It was reported that *C. violaceum* can produce polymer composed primarily of 3HB and 3-hydroxyvalerate (3HV) while being capable of accumulating poly(3-hydroxyvalerate) [P(3HV)] homopolymer when grown in valerate (Steinbüchel *et al*., 1993; Kolibachuk *et al*., 1999). *C. violaceum* is also known to accumulate 3HV and trace

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amounts of 3HHx from odd or even carbon numbered fatty acids (Kolibachuk *et al*., 1999). The complete genome of *C. violaceum* has been sequenced and annotated (Vasconcelos *et al*., 2003). The cloning and molecular analysis of the *C. violaceum* PHA synthase and heterologous expression of the cloned gene in *Escherichia coli*, *Klebsiella aerogenes*, *P. putida* (*phaC* mutant) and *Cupriavidus necator* PHB$^-$4 (*phaC* mutant) have been investigated (Kolibachuk *et al*., 1999).

In this study, a locally isolated *Chromobacterium* sp. USM2 has been evaluated for its ability to synthesize PHA, and subsequently its PHA synthase gene was cloned and expressed in *C. necator* PHB$^-$4. Unlike in the previous study whereby 3HHx was produced from fatty acids (Kolibachuk *et al*., 1999), here we demonstrate that up to 4 mol% of 3HHx could be produced by the recombinant *C. necator* PHB$^-$4 using crude palm kernel oil (CPKO) as the sole carbon source. The usage of a renewable and cheaper carbon source such as CPKO compared to fatty acids could aid in reducing the overall production cost of P(3HB-*co*-3HHx). Besides this, CPKO is mainly used in the oleochemical industries, thus the bioconversion of CPKO to PHA will not affect the food industry.

MATERIALS AND METHODS

Isolation and characterization of *Chromobacterium* sp. USM2

Fresh water samples from 4 different locations in the northern peninsular of Malaysia were obtained. The samples were plated on nutrient rich (NR) agar (Doi *et al*., 1995) after serial dilution and incubated at room temperature for 24–48 h. Visible colonies were picked and purified by re-culturing to obtain single colonies. Subsequently, the isolates were screened for PHA production via one-stage cultivation. Isolates were precultured in 50 mL NR medium. Growth of the cells was monitored by determining the optical density (OD) at the wavelength of 600 nm and upon reaching OD_{600nm} of 3.0, 3 % (v/v) of the inoculum was transferred into Schlegel's medium consisting of the following components: per liter; 9.0 g of $Na_2HPO_4{\cdot}12H_2O$, 1.5 g of KH_2PO_4, 0.1 g of NH_4Cl, 0.2 g of $MgSO_4{\cdot}7H_2O$, 0.02 g of $CaCl_2{\cdot}2H_2O$, 0.0012 g of $C_6H_8O_7{\cdot}Fe(III){\cdot}NH_3$ and 10 mL/L of trace element solution consisting of the following components: per liter of 0.1 M HCL; 500 mg of EDTA, 200 mg of $FeSO_4{\cdot}7H_2O$, 10 mg of $ZnSO_4{\cdot}7H_2O$, 3 mg of $MnCl_2{\cdot}4H_2O$, 30 mg of H_3BO_3, 20 mg of $CoCl_2{\cdot}6H_2O$, 1 mg of $CuCl_2{\cdot}2H_2O$, 2 mg of $NiCl_2{\cdot}6H_2O$ and 3 mg of $Na_2MoO_4{\cdot}2H_2O$. The culture was supplemented with 10 g/L of glucose. Cultivation was carried out in 50 mL of Schlegel's medium in 250 mL conical flask and incubated at 30 °C at 150 rpm on a reciprocal shaker. PHA production was screened by observing PHA granules in the cells using 1 % (w/v) Nile Blue A staining method (Ostle and Holt, 1982). In addition, biochemical characterization and 16S RNA sequencing was carried out to identify and characterize the isolated bacterium. The API 20 NE kit (Biomérieux Co.) was used according to the protocol supplied by the manufacturer. Isolation of genomic DNA (gDNA) was carried out as described by Sambrook *et al*. (1989). The gDNA was then used as template for PCR amplification of the 16S rRNA fragment. The PCR product obtained was gel purified with the QIAquick (Qiagen, USA) and sequenced at First Base Laboratory (Biosyntech), Malaysia. The nucleotide sequence was compared with those in the GenBank database by using the National Centre for Biotechnology Information (NCBI) Blast program.

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* was grown at 37 °C in Luria-Bertani medium consisting of the following components: per liter; 10 g casein enzymic hydrolysate, 5 g yeast extract and 10 g NaCl at pH 7.0 while *Chromobacterium* sp. USM2 and *C. necator* PHB$^-$4 were grown at 30 °C in NR medium. Cultures were incubated on a reciprocal shaker at 200 rpm. Kanamycin (50 mg/L) was added when necessary to maintain plasmid stability. For maintenance purpose, bacterial cultures from the exponential growth phase were stored at –20 °C in 20% (v/v) glycerol.

For PHA biosynthesis, two-stage cultivation was employed. *Chromobacterium* sp. USM2 strain was first grown in 50 mL NR medium to increase cell biomass. After 24 h of cultivation, cells were harvested and transferred aseptically after rinsing twice with sterile distilled water into Schlegel's medium supplemented with various carbon sources. Sugars such as glucose (10 g/L), fructose (10 g/L) and the salt form of 3HV-precursors namely sodium valerate (10 g/L) and sodium propionate (5 g/L) were filter-sterilized. Meanwhile, valeric acid (1 g/L), hexanoic acid (10 g/L) and CPKO (5 g/L) which is rich in saturated and unsaturated fatty acids were sterilized by autoclaving. The carbon sources were added during inoculation according to their respective concentrations. Cultures were grown at 30 °C at 200 rpm for 48 h. After 48 h of cultivation, cells were harvested by centrifugation (6000 x *g*, 4 °C for 5 min). After being washed with hexane to remove residual oils (for culture supplemented with CPKO), the cells were rinsed with distilled water before lyophilization. Lyophilized cells were subjected to methanolysis for 140 min at 100 °C in the presence of methanol/sulfuric acid (85:15 v/v). The total amount of PHA and its composition were determined by using gas chromatography (GC) with caprylic methyl ester as an internal standard (Braunegg *et al*., 1978). Total cell biomass was determined by measuring cell dry weight (CDW).

DNA manipulation, plasmid construction and nucleotide sequence analysis

Isolation of total genomic DNA (gDNA) from *Chromobacterium* sp. USM2, plasmid DNA isolation, agarose gel electrophoresis and transformation of *E. coli* were carried out according to standard procedures

Table 1: Bacterial strains and plasmids used in this study

Bacterial strains and plasmids	Relevant phenotype	Source or reference
Bacterial strains		
Escherichia coli		
JM109	E14–(*mcr*A), *rec*A1, *gry*A96, *thi*-1, *hsd*R17(rk-, mk+), *sup*E44, *rel*A1, D(*lac-pro*AB), [F' *tra*D36, *pro*AB, *lac*I^{q}ZΔM15]	Stratagene
S17-1	*rec*A and *tra* genes of plasmid RP4 integrated into chromosome; auxotrophic for praline and thiamine	Simon *et al*., 1983
Chromobacterium sp. USM2	Wild type	This study
Cupriavidus necator		
H16	Wild type	ATCC 17699
PHB$^-$4	PHA-negative mutant of H16	DSM 541
Plasmids		
pGEM-T easy	*rec*A, *end*A1, *gyr*A96, *thi*, hsdR17(r_{K-}, m_{K+}), relA1, *sup*E44, Δ(*lac-pro*AB), [F', *tra*D36, *pro*AB, *lac*I^{q}ZΔM15]	Promega
pBBR1MCS-2	Km^r, broad host range, *lac* POZ'	Kovach *et al*., 1995
pGEM-C2	pGEM-T easy vector derivative harboring approximately 2.0 kb fragment of *phaC* from *Chromobacterium* sp. with putative promoter	This study
pBBR1MCS-C2	pBBR1MCS-2 derivative harboring approximately 2.0 kb fragment of *phaC* from *Chromobacterium* sp. with putative promoter	This study

(Sambrook *et al*., 1989). Restriction enzymes were used according to the manufacturers' protocols such as Promega and New England Biolabs, respectively. The whole gDNA of *Chromobacterium* sp. USM2 was used as the template for PCR amplification of its PHA synthase gene (*phaC*$_{Cs}$) using a pair of domain-specific primers designed using NCBI as a reference. Following are the sequences of the primers used; Forward primer (F1): 5´-cgtaattggggcccatgcag-3´ and Reverse primer (R1): 5´-agccgccgccgaagcttccgatggc-3´. PCR was carried out using Peltier Thermal Cycler (PTC-200). The amplified fragment was purified using SpinClean™ Gel Extraction Kit. The purified fragment approximately 2.0 kb was then ligated into pGEM-T easy cloning vector (Promega) according to the manufacturer's protocol. The derived pGEM-C2 plasmid was then digested with *Apa*I and *Sal*I to obtain *phaC*$_{Cs}$ with sticky ends. The digested product was then introduced into the *Apa*I-*Sal*I digested sites of the broad-host-range vector pBBR1MCS-2 (Kovach *et al*., 1995). Hence, the pBBR1MCS-C2 plasmid was constructed for the functional expression of *phaC*$_{Cs}$ in *C. necator* PHB$^-$4. DNA sequencing was carried out by the dideoxy chain termination method with the Prism 310 DNA sequencer (Applied Biosystems, Inc.) employing the dye terminator labeling procedure (Perkin Elmer Corp.).

Functional expression of *Chromobacterium* sp. USM2 PHA synthase gene in *C. necator* PHB$^-$4

Plasmid pBBR1MCS-C2 was introduced into *C. necator* PHB$^-$4 as described in (Friedrich *et al*., 1981). For PHA biosynthesis, one-stage cultivation was employed. *C. necator* PHB$^-$4 strain harboring pBBR1MCS-C2 was first precultured in NR medium (Doi *et al*., 1995) for 12 h before being transferred (3 % [v/v]) into a 250 mL flask containing 50 mL mineral medium (MM) (Doi *et al*., 1995). *C. necator* strains are known to grow and produce PHA from carbon substrates such as sugars or plant oils. Hence, fructose and CPKO were chosen as carbon substrates for PHA production using this recombinant. CPKO or fructose was added to the culture medium at a final concentration of 5 g/L when fed as the sole carbon source and 2.5 g/L respectively in the presence of precursor. Sodium valerate (2.5 g/L) was added at 24 h as precursor for the generation of 3HV monomer. Kanamycin (50 mg/L) was added to maintain plasmid stability. Cultures were grown at 30 °C at 200 rpm for 48 h. After 48 h of cultivation, the cells were harvested, lyophilized and subjected to GC analysis as mentioned earlier.

Nuclear Magnetic Resonance (NMR)

The PHA accumulated in the cells was first extracted by refluxing lyophilized cells with chloroform for 4 h at 60 °C. After filtration, the chloroform extract was concentrated

and the dissolved PHA was precipitated in chilled methanol. A total of 25 mg of PHA sample was dissolved in 1 mL of deuterated chloroform ($CDCl_3$). The ^1H NMR spectrum was measured on a Bruker AVANCE 300; NC, USA spectrometer at 400 MHz at 30 °C. Tetramethylsilane (Me_4Si) was used as an internal chemical shift reference.

Electron microscopic analysis (TEM)

TEM analysis was carried out to observe the accumulation of PHA granules and the changes in cell morphology under the electron microscope (Philip CM 12/ STEM and JLM-2000FX11). Cells were harvested and fixed in McDowell-Trump fixative at 4 °C for 24 h (McDowell and Trump, 1976). The cell pellets were then post-fixed with 1% osmium tetroxide (OsO_4) at room temperature. Cells were dehydrated in an increasing ethanol series (50, 75, 95 and 100%) and then transferred to 100% acetone. Cells were embedded at 60 °C for 24-48 h in Spurr's low viscosity resin (Spurr, 1969). Ultra-thin sections were prepared, mounted on copper grids and stained with uranyl acetate and lead citrate for electron microscope examination at an acceleration voltage of 80 kV (Philip CM 12/ STEM and JLM-2000FX11).

RESULTS AND DISCUSSION

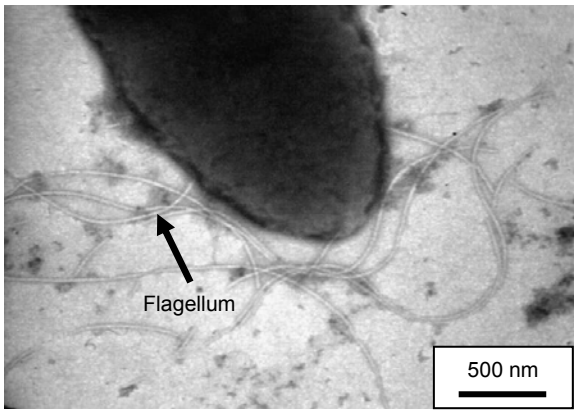

Figure 1: TEM image showing the presence of flagella on *Chromobacterium* sp. USM2

Isolation and screening of PHA producing strain

Eight bacterial isolates were obtained from the fresh water samples which were spread on NR agar plates. These isolates were grown in Schlegel's medium which was supplemented with 10 g/L of glucose. Among the cultured isolates, only one which was obtained from the Seven Wells waterfall, Langkawi Island emitted bright orange fluorescence with Nile Blue A under fluorescence microscope. This suggests the presence of PHA-like granules in the bacterium. Morphological and biochemical tests were done to identify the isolate. Colonies on the NR agar were violet pigmented, circular with entire edge, convex and the surface was smooth and glistering. The diameter of the colonies ranged between 0.5 mm to 1.0 mm. Meanwhile, the cells were motile by means of flagella (Figure 1), non-spore forming and rod-shaped (1.5 µm-3 µm × 0.4 µm-0.5 µm). According to the biochemical tests, the isolate was determined as Gram negative, non-lactose fermenting aerobic bacterium. Optimum growth was observed at 30 °C. The isolate was then identified as *C. violaceum* with an accuracy of 99.9% based on the API 20 NE kit results which were interpreted using Apiweb™ stand alone V1.2.1 software. The partial 16S rRNA sequence (GenBank accession no. FJ668944) further confirmed this with 98% similarity to *C. violaceum*. The isolate was denoted as *Chromobacterium* sp. USM2 and deposited in Japanese Culture Collection with the accession number of JCM 15051.

Nucleotide sequence of *Chromobacterium* sp. USM2 PHA synthase gene

The *phaC*$_{Cs}$ consisting of 1704 nucleotides was successfully cloned using the pair of domain-specific primers. The gene encoded a protein of 568 amino acids which is the PHA synthase, PhaC. The Shine-Dalgarno (S/D) sequence GGAAGA was located 37 nucleotides upstream of the start codon ATG. According to the *C. violaceum* genome project (Brazilian Genome, 2009), there are six pairs of identified promoters in the *phaC* gene of *C. violaceum*. The probable promoter pair in *phaC*$_{Cs}$ was located 57 bp upstream of the start codon with the sequence of (GTTTCA-17N-CAGAAT), which was identified as -35 and -10 region respectively. TGA was the stop codon for this gene. The *phaC*$_{Cs}$ sequence was aligned with various known PHA producers such as; *Aeromonas caviae, Alcaligenes latus, Burkholderia* sp. DSMZ9242, *C. violaceum, C. necator*, *Delftia acidovorans, Paracoccus denitrificans* and *Streptomyces aureofaciens* using Clustal X. The alignment formed 3 clustals and it was found that *phaC*$_{Cs}$ of the isolated *Chromobacterium* sp. USM2 was in the same clustal as *D. acidovorans* with similarity of 84%. It is interesting to note that the *phaC*$_{Cs}$ is in a different clustal from *C. violaceum* even though it has high similarity of 89%.

The *phaC*$_{Cs}$ was successfully introduced into the broad-host-range vector pBBR1MCS-2 for the construction of pBBR1MCS-C2 plasmid which was later used for functional expression of *phaC*$_{Cs}$ in *C. necator* PHB$^-$4.

PHA accumulation by *Chromobacterium* sp. USM2 from various carbon sources

The ability of *Chromobacterium* sp. USM2 to synthesize PHA from various carbon sources was investigated (Table 2). It has been reported that *C. violaceum* produces poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)] copolymer when glucose or fructose was fed as sole carbon source (Kolibachuk *et al*., 1999). However, *Chromobacterium* sp. USM2 only accumulated P(3HB)

Table 2: Biosynthesis of PHA by *Chromobacterium* sp. USM2 from various carbon sources[a]

Carbon sources	Cell dry weight (g/L)	PHA content[b] (wt%)	PHA compositions (mol%)	
			3HB	3HV
Glucose	3.0 ± 0.2	22 ± 4	100	0
Fructose	2.8 ± 0.2	14 ± 2	100	0
Valeric acid	2.9 ± 0.1	10 ± 2	12	88
Hexanoic acid	2.7 ± 0.2	2 ± 1	100	0
Sodium valerate	3.1 ± 0.2	32 ± 2	2	98
Sodium propionate	2.8 ± 0.1	2 ± 1	62	38
CPKO	3.0 ± 0.2	23 ± 4	100	0

3HB, 3-hydroxybutyrate; 3HV, 3-hydroxyvalerate
[a]Incubated for 48 h at 30 °C, initial pH 7.0, 200 rpm in Schlegel's medium.
[b]PHA content in freeze-dried cells

homopolymer up to 22 ± 4 wt% and 14 ± 2 wt% from glucose and fructose, respectively. *C. violaceum* is also known to accumulate 3HV and trace amounts of 3HHx in saturated and unsaturated fatty acids (Loo *et al*., 2005) to determine the possibility of 3HV and 3HHx from odd or even carbon numbered fatty acids (Kolibachuk *et al*., 1999). Therefore, *Chromobacterium* sp. USM2 was fed with hexanoic acid and CPKO which is rich incorporation. However, it was found that *Chromobacterium* sp. USM2 could not incorporate neither 3HV nor 3HHx monomers from hexanoic acid or CPKO. The bacterium accumulated only 23 wt% of P(3HB) from CPKO while a very low amount of P(3HB) was produced with hexanoic acid (Table 2). The results suggested that the *Chromobacterium* sp. USM2 has PHA synthesizing abilities that differ slightly from that of the *C. violaceum* reported by Kolibachuk *et al*. (Kolibachuk *et al*., 1999).

P(3HB-*co*-3HV) copolymer was successfully produced by cultivating *Chromobacterium* sp. USM2 in known 3HV-precursors such as sodium valerate, sodium propionate or valeric acid as the sole carbon source. Based on the results shown in Table 2, the effect of the carbon sources on PHA content and 3HV composition could be arranged in the following order: sodium propionate < valeric acid < sodium valerate. Higher PHA content (32 ± 2 wt%) and 3HV composition (98 mol%) was obtained when the isolate was fed with 1.0% sodium valerate. Sodium valerate was the better precursor for high 3HV generation. This observation was consistent with the biosynthetic pathways where activated valerate (valeryl-CoA) could be directly incorporated as a monomeric unit since propionyl-CoA instead of acetyl-CoA is the final product of the β-oxidation cycle (Steinbüchel and Lütke-Eversloh, 2003).

According to Kolibachuk and co-workers, *C. violaceum* accumulated highest 3HV monomer composition of 95 mol% with a copolymer content of 15 wt% from heptanoic acid (Kolibachuk *et al*., 1999). In this study, *Chromobacterium* sp. USM2 was found to accumulate 98 mol% of 3HV from sodium valerate with a copolymer content of 32 ± 2 wt%. However, in the presence of valeric acid, the 3HV composition and PHA content were lower at 88 mol% and 10 ± 2 wt%, respectively. In a previous study by Park and co-workers,

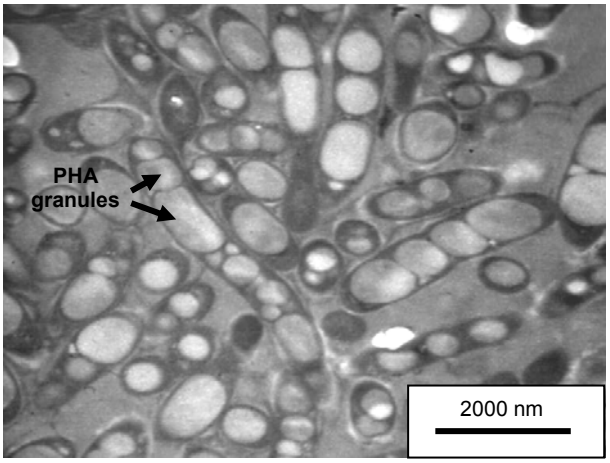

Figure 2: TEM image of *C. necator* PHB⁻4 harboring *phaC*$_{Cs}$ containing P(3HB) granules after 48 h cultivation with 5 g/L of fructose at 30 °C.

it was found that valeric acid exhibited some level of inhibitory effect on cell growth (Park *et al*., 2001). The toxicity effect of the valeric acid was reduced by converting it from acid form to its salt form according to Loo and Sudesh (Loo and Sudesh, 2007b). Different concentrations of sodium valerate ranging from 5 g/L to 15 g/L were fed to the bacterial culture in order to determine the effects on 3HV composition and copolymer production. However, the varying concentrations did not register any significant changes on the 3HV molar fraction. Higher concentrations of sodium valerate may have exerted some toxic effect on the bacterial culture since there was a decrease in cell biomass and PHA content (results not shown).

The type of PHA produced by a certain microorganism is determined by not only the PHA synthase but also the metabolic pathways of that microorganism. In order to determine the ability of the *Chromobacterium* sp. USM2 PHA synthase to incorporate other monomers, *phaC*$_{Cs}$ was expressed in *C. necator* PHB⁻4.

Table 3: Biosynthesis of PHA by *C. necator* PHB⁻4 harboring the PHA synthase gene of *Chromobacterium* sp. USM2[a]

Carbon sources	Strains	Cell dry weight (g/L)	PHA content (wt%)[b]	PHA compositions (mol%)		
				3HB	3HV	3HHx
Fructose	*C. necator* PHB⁻4 (pBBR1MCS-C2)	3.1 ± 0.2	64 ± 2	100	-	-
	C. necator H16	3.3 ± 0.1	56 ± 1	100	-	-
	C. necator PHB⁻4 (pBBR1MCS-2)	2.3 ± 0.2	0	-	-	-
Fructose + Sodium valerate	*C. necator* PHB⁻4 (pBBR1MCS-C2)	2.8 ± 0.2	57 ± 2	40	60	-
	C. necator H16	3.0 ± 0.1	38 ± 2	65	35	-
CPKO	*C. necator* PHB⁻4 (pBBR1MCS-C2)	4.0 ± 0.2	63 ± 2	96	-	4
	C. necator H16	5.1 ± 0.4	60 ± 1	100	-	-
CPKO + Sodium valerate	*C. necator* PHB⁻4 (pBBR1MCS-C2)	3.7 ± 0.3	42 ± 2	87	9	4
	C. necator H16	4.8 ± 0.3	40 ± 3	91	5	

3HB, 3-hydroxybutyrate; 3HV, 3-hydroxyvalerate; 3HHx, 3-hydroxyhexanoate
[a]Incubated for 48 h at 30 °C, initial pH 7.0, 200 rpm in MM. Sodium valerate was added at 24 h of cultivation.
[b]PHA content in freeze-dried cells

Expression of *Chromobacterium* sp. USM2 PHA synthase gene in *C. necator* PHB⁻4

Expression of the cloned *phaC*$_{Cs}$ in *C. necator* PHB⁻4 complemented the recombinant strain and resulted in accumulation of PHA. The yields and PHA composition produced by the recombinant strain were compared with that of the wild type *C. necator* H16 (Table 3). The recombinant *C. necator* PHB⁻4 harboring *phaC*$_{Cs}$ could utilize fructose for the production of P(3HB) homopolymer. The CDW (3.1 ± 0.2 g/L) and PHA content (64 ± 2 wt%) was almost similar to that of wild type *C. necator* H16 (3.3 ± 0.1 g/L and 56 ± 1 wt%). The TEM micrograph in Figure 2 further confirms the formation of PHA granules in the recombinant. The bacterium was packed with large quantities of distinctly visible granules, resulting in the cells to appear elongated. As expected, no PHA accumulation was observed in *C. necator* PHB⁻4 harboring only the plasmid pBBR1MCS-2 without the synthase gene. Higher CDW was obtained when CPKO was used as the sole carbon source. The cell biomass of recombinant harboring *phaC*$_{Cs}$ was 4.0 ± 0.2 g/L. However, the PHA content was almost similar as when fructose was used.

Interestingly, in the presence of CPKO, accumulation of P(3HB-*co*-3HHx) copolymer with 4 mol% of 3HHx was observed in the recombinant *C. necator* PHB⁻4 harboring *phaC*$_{Cs}$. This was not evident in the wild type *Chromobacterium* sp. USM2. Previously, Kolibachuk and co-workers noticed that 3HHx monomer was accumulated by recombinant *C. necator* PHB⁻4 harboring the PHA synthase gene of *C. violaceum* when fed with even carbon numbered fatty acids (Kolibachuk *et al*., 1999). Similar studies were also done to generate 3HHx monomer by expressing PHA synthase gene of *A. caviae* in mutant *C. necator* PHB⁻4 (Fukui and Doi, 1997). *A. caviae* is known to synthesize both SCL and MCL PHA containing four to six carbon atoms (Shimamura *et al*., 1994). Various plant oils including palm oil products have been used to produce P(3HB-*co*-3HHx) copolymer from the recombinant *C. necator* PHB⁻4 harboring the PHA synthase gene of *A. caviae* (Fukui and Doi, 1998; Kahar *et al*., 2004; Loo *et al*., 2005). The generation of a new monomer in a different host could be associated with the occurrence and substrate specificity of enzymes. The expression of a foreign gene in a microorganism might result in the formation of additional enzymes or same enzymes with different substrate specificity towards the expressed gene compared to that of in the wild type microorganism. Besides this, availability of additional metabolic pathway in the new host might influence the production of PHA and its monomers. The factors mentioned above might have facilitated the incorporation of 3HHx monomer when *phaC*$_{Cs}$ was expressed in *C. necator* PHB⁻4 using its native *phaA* and *phaB* genes.

To investigate the production of P(3HB-*co*-3HV) copolymer, sodium valerate was added to the culture supplemented with fructose (Table 3). The 3HV composition generated by the recombinant harboring *phaC*$_{Cs}$ gene was nearly 2-fold higher compared to the wild-type *C. necator*. The PHA content produced by this recombinant was also higher at 57 ± 2 wt% compared to

the wild type (38 ± 2 wt%). These results indicated the affinity towards the incorporation of 3HV by the PHA synthase of *Chromobacterium* sp. USM2. High 3HV composition has been achieved using wild-type *C. necator* from selected 3HV-precursors or mixtures of these precursors with sugar. However, the high 3HV composition is mostly achieved by feeding higher concentration of precursors, up to 4-20 g/L (Doi *et al*., 1988; Ramsay *et al*., 1990; Madden *et al*., 1998; Volova and Kalacheva, 2005). The ability of the PHA synthase of *Chromobacterium* sp. USM2 to accumulate high amount of 3HV from lower concentration of precursor (2.5 g/L of

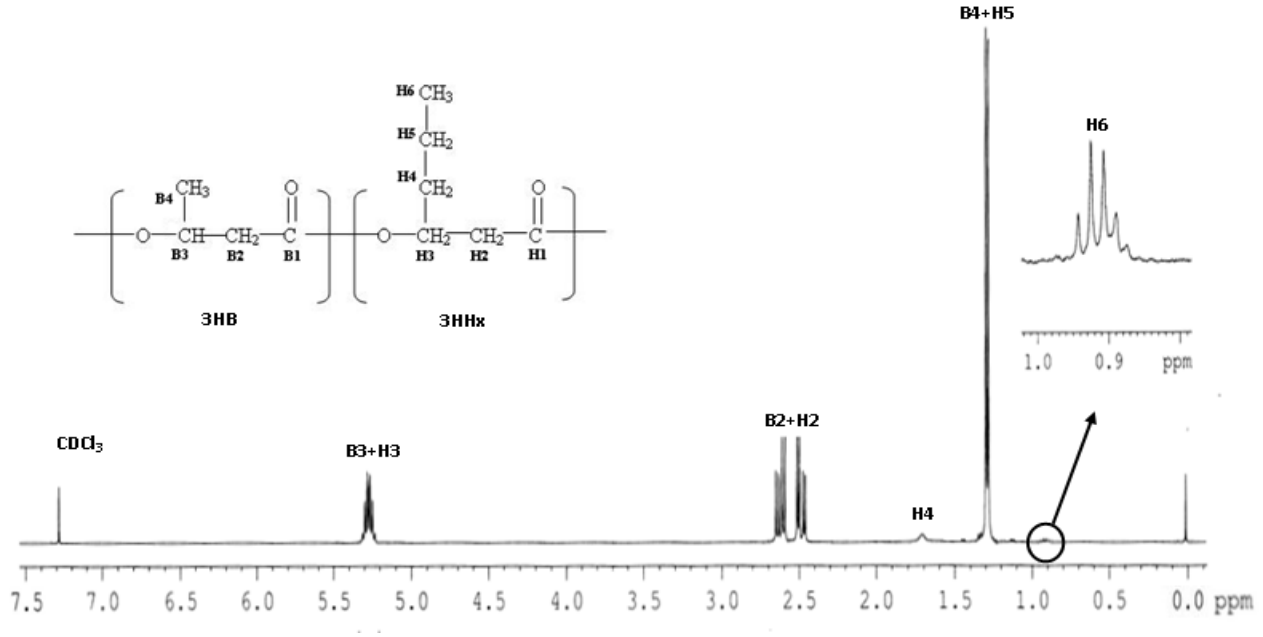

Figure 3: ^{1}H NMR spectrum confirming the presence of 3HHx monomer in P(3HB-*co*-3HHx) copolymer produced by *C. necator* PHB^{-}4 harboring *phaC*$_{Cs}$ from CPKO

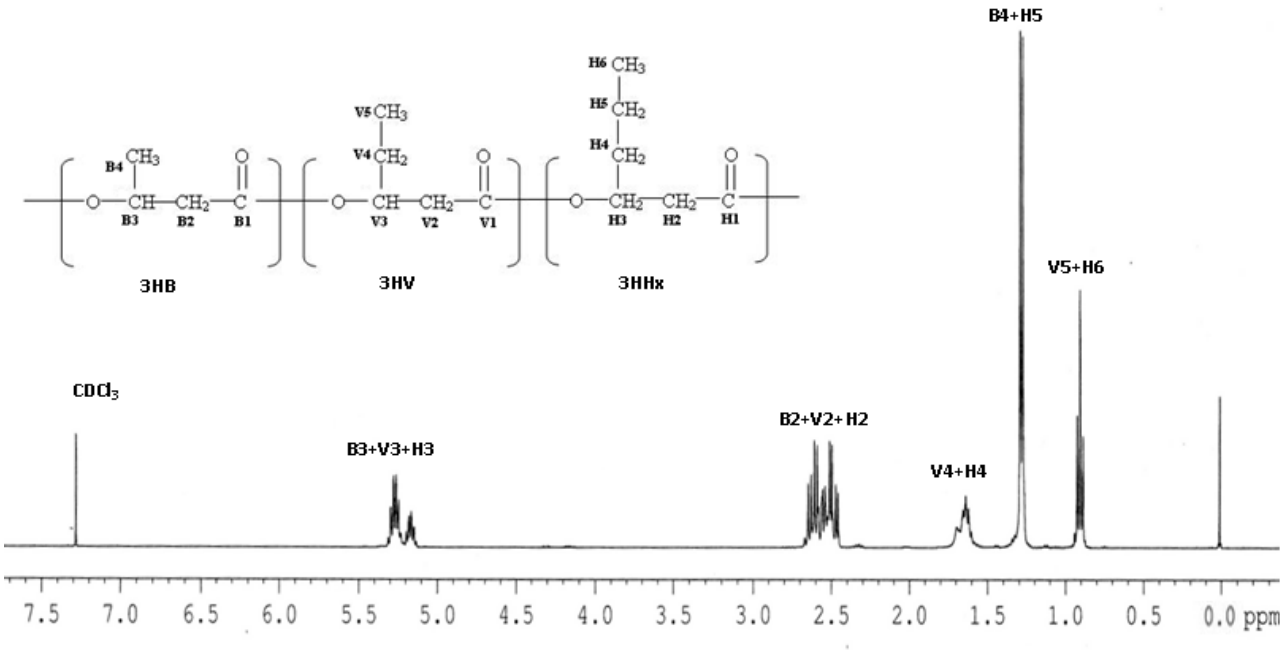

Figure 4: ^{1}H NMR spectrum confirming the presence of 3HV and 3HHx monomers in P(3HB-*co*-3HV-*co*-3HHx) terpolymer produced by *C. necator* PHB^{-}4 harboring *phaC*$_{Cs}$ from mixture of CPKO and sodium valerate

sodium valerate) could reduce inhibitory effects towards cell growth and polymer accumulation.

Addition of sodium valerate in the presence of CPKO generated poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HV-*co*-3HHx)] terpolymer. Previously, production of P(3HB-*co*-3HV-*co*-3HHx) terpolymer had been investigated from palm kernel oil and mixtures of sodium valerate or propionate by using recombinant *C. necator* PHB^{-}4 harboring the PHA synthase of *A. caviae* (Bhubalan *et al*., 2008). The terpolymers were found to have interesting properties due to the incorporation of both 3HV and 3HHx monomers. Similar study was also done by Park and coworkers whereby the PHA biosynthesis genes of *Aeromonas* were heterologously expressed in recombinant *E. coli* (Park *et al*., 2001). They used dodecanoic acid plus odd carbon number fatty acid for the generation of P(3HB-*co*-3HV-*co*-3HHx) terpolymer.

This recombinant was able to produce 42 ± 2 wt% of polymer containing 87 mol% 3HB, 9 mol% 3HV and 4 mol% 3HHx, respectively. It was found that, the 3HV monomer composition generated in the presence of CPKO was lower compared to when fructose was used. This could be explained by the different monomer biosynthesis pathways that are being used to convert the substrates into PHA. In the presence of a mixture of fructose and sodium valerate, only sodium valerate enters the β-oxidation pathway for the synthesis 3HV monomer. However, both CPKO and sodium valerate simultaneously enter the β-oxidation pathway for the synthesis of 3HB, 3HV and 3HHx monomers (Bhubalan *et al*., 2008). Hence, the generation of 3HV monomer might be affected due to the competition in monomer synthesis. The 3HV composition produced by this recombinant from mixture of CPKO and sodium valerate was almost similar with the value reported by Lee and co-workers during P(3HB-*co*-3HV) copolymer production using wild-type *C. necator* and the same mixture of carbon source (Lee *et al*., 2008). However, in this study only half the amount of sodium valerate (2.5 g/L) was used compared to the initial concentration of 5 g/L which was used by Lee *et al*. (2008).

Nevertheless, it is possible to attain terpolymers with a wider range of 3HV monomer composition. The 3HV monomer composition in P(3HB-*co*-3HV-*co*-3HHx) terpolymers are known to be varied by altering the concentration of 3HV-precursor and its feeding time (Bhubalan *et al*., 2008). Prolonged cultivation period might also enable a more efficient conversion of precursor substrate to 3HV monomer as in this study the cultures were only incubated for 48 h. Therefore, controlled synthesis of P(3HB-*co*-3HV-*co*-3HHx) terpolymers using this recombinant could be further investigated in future studies.

In order to further verify the presence of 3HHx in the copolymer and 3HV and 3HHx monomers in the terpolymer chain, ^{1}H NMR analysis was carried out. From the spectra in Figures 3 and 4, the presence of 3HB, 3HV and 3HHx monomers were identified. Methyl proton resonance at 0.9 ppm can be assigned to CH_3 group from 3HV and 3HHx monomers. In addition, CH_2 group at 1.7 ppm also indicates the presence of 3HV and 3HHx monomers in the terpolymer chain. The presence of 3HV monomer in the terpolymer could be correlated with an increase in the intensity of this peak. ^{1}H NMR results further confirmed the findings of GC analysis.

CONCLUSIONS

In this study, *Chromobacterium* sp. USM2 a locally isolated bacterium was found to be capable of accumulating P(3HB-*co*-3HV) with high 3HV monomer composition. Its PHA synthase gene was successfully cloned and heterologously expressed in *C. necator* PHB^{-}4. Accumulation of P(3HB-*co*-3HHx) copolymer was identified once the recombinant was supplemented with CPKO as the sole carbon source. In addition, the recombinant was also capable of biosynthesizing P(3HB-*co*-3HV-*co*-3HHx) terpolymer when a mixture of sodium valerate and CPKO was fed. Further study is needed to understand the ability of both the isolate and recombinant in the production of different types of PHA. The resulting polymers could be tailored and characterized to suit selected applications.

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