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Engineering of β-oxidation pathway to tailor polyhydroxyalkanoate (PHA) production in *Bacillus thermoamylovorans*

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ABSTRACT: Polyhydroxyalkanoate (PHA) is a type of microbial polyester that is stored within cells as a carbon and energy source. This study evaluated the effects of knockout gene mutation on the production and modification of PHA in *Bacillus thermoamylovorans*. The genes *fad*B and *fad*A, which encode 3-ketoacyl-CoA thiolase and 3-hydroxyacyl-CoA dehydrogenase, respectively, in *B. thermoamylovorans* were deleted to suppress the β-oxidation pathway. The potential of the wild type and mutant to produce PHA under various fatty acids was subsequently studied. The wild type produced the highest biomass and PHA content, measuring 4.5 ± 0.3 g/l and 41.4 ± 1.0%, respectively, when grown on sodium octanoate. The similar pattern was observed in mutant strain, which yielded 3.8 ± 0.2 g/l of biomass and 41.0 ± 1.0% PHA under the same condition. In addition, heterogeneous PHA was detected in both the wild-type and mutant strains. These findings indicate that *fad*B and *fad*A are crucial for fatty acid degradation. However, deleting *fad*B and *fad*A did not negatively affect biomass or PHA production. Furthermore, the production of PHA was scaled up in a 3-l fermenter. High values of the specific growth rate, biomass productivity, and maximum productivity were detected in the wild-type. Interestingly, the mutant strain produced medium-co-long-chain-length (mcl-co-lcl) PHA, whereas the wild-type strain primarily synthesized short-chain-length (scl) PHA. Compared with scl-PHA, mcl-PHA is more advantageous because of its superior elasticity, reduced crystallinity and tensile strength, and elevated melting point. This study represents the first report of enhanced PHA production in *B. thermoamylovorans* through knockout of the *fad*B and *fad*A genes.

KEYWORDS: *Bacillus thermoamylovorans*, β-oxidation, knockout mutation, polyhydroxyalkanoate, waste cooking oil

INTRODUCTION

PHA is a biodegradable polyester produced by various microorganisms, typically under stressful environmental conditions [1]. These stress conditions can include nutrient limitations (like nitrogen, phosphorus, or oxygen) or other environmental stressors such as extreme temperatures, oxidative stress, and UV radiation [2]. The properties of PHA resemble those of petroleum-based polymers such as polypropylene (PP) and polyethylene (PE) [3]. However, its applications are restricted by its chemical properties [4]. PHA monomers are categorized into three types based on their carbon chain length: scl- (4–5 carbon atoms), mcl (6–14 carbon atoms) and lcl (> 15 carbon atoms) [4, 5]. The scl-PHA is the most frequently used material in various fields. However, scl-PHA exhibits poor mechanical properties, similar to those of hard and brittle thermoplastics, with high melting temperatures, low thermal stability, and high crystallinity indexes [4]. The mcl- and lcl-PHA have better properties than regular elastomers, good elongation properties, low melting temperatures and low crystallinities [6]. Nevertheless, the high production and operation costs are the greatest for mcl- and lcl-PHA production. Using inexpensive substrates, such as urban or industrial wastewater [7] and waste cooking oil (WCO), is an effective way to lower the production costs of PHA significantly.

WCO, a municipal waste, is largely produced by households and restaurants. Most WCOs are unused and directly discharged into the environment without proper treatment [8]. According to the U.S. Environmental Protection Agency (EPA), 15 million tons of WCO are generated each year [8]. Therefore, the use of WCO has become the main consideration in overcoming WCO management. To our knowledge, *B. thermoamylovorans* PHA005 produces a high PHA yield of 3.5 g/l, accounting for 87.5% of the cell dry mass (CDM) when 4–5% (w/v) WCO is used as the substrate. Moreover, this strain demonstrated a strong ability to grow under high concentrations of WCO, reaching as high as 50% (w/v). The PHA produced by *B. thermoamylovorans* PHA005 was identified as poly(3-hydroxybutyrate-co-3-hydroxyvalerate), with 85 mol% 3-hydroxybutyrate (3HB) and 15 mol% 3-hydroxyvalerate (3HV) [9]. However, PHA production using WCO remains low in *B. thermoamylovorans*

because of the limited substrate affinity of the β-oxidation pathway. This pathway is crucial for producing intermediates for PHA synthesis when fatty acids or oils are used as the sole carbon source [10]. In the β-oxidation pathway, fatty acids are broken down and converted into enoyl-CoA. Ultimately, R-3-hydroxyacyl-CoA and mcl-PHA are formed through the enzymatic activity of R-3-hydroxyacyl-CoA hydratase and PHA synthase, respectively [4]. Two key genes, *fad*B and *fad*A, are involved in β-oxidation. These genes encode 3-ketoacyl-CoA thiolase and 3-hydroxyacyl-CoA dehydrogenase, respectively [10]. The improvement of PHA by β-oxidation mutation was reported in *Pseudomonas putida* KT2442 [11]. The *P. putida* mutant produced high PHA (84% CDM), while *fad*B and *fad*A were deleted. In addition, a high 3-hydroxydodecanoate (3HDD) monomer in PHA (41 mol%) has also been reported. The parent strain of *P. putida* yielded only 50% CDM and 7.5 mol% PHA and HDD fractions, respectively [11]. The connection between PHA production and β-oxidation was investigated by knocking out the genes encoding 3-ketoacyl-CoA thiolase, 3-hydroxyacyl-CoA dehydrogenase and acetyl-CoA acetyltransferase in *Pseudomonas entomophila*. The mutant strain accumulated PHA from more than 90% CDM with 99 mol% HDD [9]. Compared with scl-PHA and conventional mcl-PHA, high 3HDD monomer (> 40 mol%) exhibits good thermal and mechanical properties [12, 13]. However, *P. putida* did not grow well after gene deletion. In addition, no PHA production was previously observed when WCO was performed with a β-oxidation mutant. Therefore, this study aimed to enhance the PHA production and function of *B. thermoamylovorans* by deleting the *fad*B and *fad*A genes, which are involved in the β-oxidation pathway. Subsequently, the effects of cultivation conditions on the mutants were investigated using various fatty acids and WCO. The production and characterization of PHA from the mutant were evaluated compared with those from the wild-type.

MATERIALS AND METHODS

Bacterial strains and culture media

The proprietary, wild-type strain *B. thermoamylovorans* PHA005, a bacterium that produce PHA, was maintained in-house at Thaksin University, Thailand. The strain PHA005 was utilized in all the experiments of this study. New plates were prepared from vials that had been stored at −80 °C. The parent strain was maintained on PHA-producing agar, which contained 2 g/l $(NH_4)_2SO_4$, 13.3 g/l KH_2PO_4, 1.2 g/l $MgSO_4 \cdot 7\,H_2O$, 1.7 g/l citric acid, 15 g/l agar, and 10 ml/l trace elements. A trace element solution was prepared by combining 1 mM each of $FeSO_4 \cdot 4\,H_2O$, $CaCl_2 \cdot 2\,H_2O$, $MnSO_4 \cdot 4\,H_2O$, and $ZnCl_2$, with the pH adjusted to 7.0 [9]. The strain was also used as a DNA recipient via electroporation. The mutant *B. thermoamylovorans* strains were cultured in PHA-producing media supplemented with 15 mg/l kanamycin. All chemicals and antibiotics used in this study were purchased from Sigma-Aldrich (MO, USA).

E. coli XL1 blue (Agilent, CA, USA) was used for plasmid construction. The strain was subsequently grown on Luria–Bertani (LB) agar supplemented with 100 µg/ml ampicillin. LB medium supplemented with the same ampicillin concentration was used for plasmid isolation. The culture was cultivated at 37 °C, 150 rpm [14].

WCO preparation

WCO was collected from a restaurant at Thaksin University in Phatthalung, Thailand. The oil was initially filtered through a muslin cloth to remove food residues and other contaminants. Afterwards, WCO was heated to remove water. The filtered WCO contained pH and acid values of 5.11 and 4.83 mg KOH/g, respectively [9]. WCO was used as a supplemented substrate for PHA production in a 3-l fermenter.

Inoculum preparation

The inoculum was prepared from an agar plate stored at 4 °C. Next, a complete loop of bacteria was inoculated into an Erlenmeyer flask containing 50 ml of PHA-producing medium. The culture was incubated at 37 °C with shaking at 150 rpm for 24 h [9]. PHA-producing medium supplemented with 15 mg/l kanamycin was used to prepare recombinant *Bacillus* strains.

Deletion of the *fad*B and *fad*A genes via the knockout method

In this study, the *fad*B and *fad*A genes involved in β-oxidation were deleted to improve the PHA composition. The suicide vector pK18mobsacB was obtained from the American Type Culture Collection (ATCC, VA, USA). The A fragment from the *B. thermoamylovorans* PHA005 genome, encompassing the full-length sequences of *fad*B and *fad*A and the partial lengths of the upstream and downstream sequences, was amplified via polymerase chain reaction (PCR) using the following primers: 5′-ATTTCTAGAGCAGATGATGGCCTTC-3′ and 3′-CTGAAGCTTTGTAATGCCGGTATAC-5′. The primers used are underlined and contained *Xba*I and *Hin*dIII restriction sites as indicated. The 1,540 bp and 1,200 bp *fad*B and *fad*A PCR products were obtained, respectively. The PCR products and pK18mobsacB were simultaneously digested with *Xba*I and *Hin*dIII and then ligated using the same enzymes to create a new plasmid [10]. Therefore, more than 50% of *fad*B and *fad*A were deleted. All enzymes were purchased from Thermo Fisher Scientific (MA, USA).

Then, heat shock was used to transfer the plasmid into competent *E. coli* XL1 blue [14]. The plasmids were selected using the Plasmid Miniprep Kit (Thermo

Fisher Scientific). Furthermore, OneTaq® DNA polymerase (New England Biolabs, MA, USA) was used to verify the plasmids.

Electroporation of *B. thermoamylovorans*

Competent cells were prepared following the methods of Mittermair et al [14] and Höfer et al [15]. First, 1.0 g of the recombinant plasmid (excluding the *fad*B and *fad*A genes) was added to an Eppendorf tube. The tube was incubated on ice for 20 min, after which the sample was transferred to a cold electroporation cuvette. The mixture was subjected to an electroporator using a Bio-Rad electroporation (CA, USA) with the following settings: 25 μF, 200 Ω, 5 ms, and 2.5 kV/cm. Then, 1 ml of PHA-producing medium was added. The mixture was then transferred to a 15-ml test tube. The culture was incubated at 37 °C for 5 h. Then, 100 μl of the cell suspension was plated onto a PHA-producing medium supplemented with 100 μg/ml ampicillin (selective agar). The plate was incubated at 37 °C for 48 h. All colonies on selective agar were selected for the next experiment.

Selection of the PHA-producing mutant and determination of the PHA content

All the selected colonies were grown in 50 ml of PHA-producing medium for 48 h. Subsequently, all the samples were collected, dried, and analyzed using gas chromatography (Hewlett Packard GC-6890, CA, USA) equipped with an HP-INNOWAX capillary column (Agilent Technologie, CA, USA). The analysis conditions are described by Sangkharak et al [9]. The isolated strains that produced more than 40% PHA were selected for PHA fermentation.

The possibility of PHA production by knockout mutants via various fatty acids

First, the selected culture was cultured in a PHA-producing medium for 12 h. Afterwards, the cells were transferred to fatty acid-containing medium. Three different fatty acids (12 g/l), including sodium octanoate, decanoate and dodecanoate, were tested for their ability to produce PHA in mutants. Each sample was cultivated at 3 °C for 48 h. Subsequently, the cells were harvested and characterized for biomass and PHA production. The amounts of biomass and PHA were compared with those of the parent *B. thermoamylovorans* strain [9, 10]. Fatty acids including sodium octanoate, decanoate and dodecanoate were purchased from Sigma Aldrich.

Scale-up of PHA production by the mutant strain in a 3-l fermenter using WCO as a substrate

A 15-ml seed culture was prepared as described earlier and inoculated into 300 ml of PHA-producing medium, which was then incubated for 12 h under the same culture conditions. The resulting 300 ml of inoculum was then transferred to a 3-l fermenter containing 1.5 l of PHA-containing medium supplemented with 4% WCO. The cells were grown at a fixed fermentation temperature (37 °C), pH 7.0 and 30% dissolved oxygen (DO) [16]. The agitation rate (200–800 rpm) was automatically adjusted to maintain the DO concentration at 30%. The pH was adjusted using 5 M NaOH and 3 M H_2SO_4. The medium containing $(NH_4)_2SO_4$ as sole nitrogen source, and the C/N ratio was fixed at 5:1 [17].

Mutant *B. thermoamylovorans* was cultivated via a batch fermentation process. The medium supplemented with WCO was subjected to sterile conditions (120 °C, 15 min) first before being used. Trace elements and $MgSO_4$ were separately added before the experiment. The process was run for 72 h, with 10 ml of each sample collected every 12 h to determine biomass, PHA production and kinetic parameters. The amounts of biomass and PHA were compared with those of the parent *B. thermoamylovorans* strain. All the experiments were performed in triplicate. The value of each parameter was reported as the mean and standard deviation. After 72 h, both mutant and wild-type *B. thermoamylovorans* cells were harvested by centrifugation (10,000 × *g*, 5 min) and then characterized [17].

Determination of residual oil concentration

To determine the amounts of residual oil in the culture medium, residual oil was separated from the culture medium using *n*-hexane [18]. A mixture of 25 ml each of *n*-hexane and culture medium was vigorously mixed for 5 min, and then the upper *n*-hexane phase, containing the oil, was passed through a separation funnel. The oil was recovered by evaporating the *n*-hexane (Sigma Aldrich) using a rotary vacuum evaporator and subsequently weighted.

Determination of kinetic parameters

The bacterial biomass, biomass productivity, specific growth rate, and biomass yield were calculated using the following formulas [9]:

Biomass (CDM, g/l) = (CDM/10 ml) × 1,000;

Specific growth rate (μ, h^{-1}) = $(\ln x - \ln x_0)/\Delta t$;

Conversion yield of product to cell ($Y_{p/x}$, g product/g cell) = $[(P - P_0)/(x - x_0)]$;

Conversion yield of product to substrate ($Y_{p/s}$, g product/g substrate) = $[(P - P_0)/(S - S_0)]$;

Conversion yield of cell to substrate ($Y_{x/s}$, g cell/g substrate) = $[(x - x_0)/(S - S_0)]$;

Maximum (Max) biomass productivity rate (R_{xm}, g/l.h) = $\max(\Delta x/\Delta t)$;

Maximum production formation rate (R_m, g/l.h) = $\max(\Delta P/\Delta t)$,

where CDM represents cell dry mass (g); x and x_0 represent the cell concentration at the current time and the initial time (g/l), respectively; t represents time (h); P and P_0 represent the product concentration at the current time and the initial time (g/l), respectively;S and S_0 represent the substrate concentration at the current time and the initial time (g/l), respectively.

Determination of the PHA content

To quantify the PHA content, the dried cells were resuspended in 10 ml of 4% HClO and incubated at 37 °C with shaking at 150 rpm for 2 h. The mixture was then centrifuged (8,000 × g, 10 min), and the residue was collected and washed twice with 5 ml of mixed solvent (acetone:ethanol:diethyl ether, 1:1:1 ratio). PHA was extracted by centrifugation (8,000 × g, 10 min) [15]. All chemicals used for the determination of the PHA content were purchased from Sigma Aldrich. The PHA content was then calculated using the following formula:

> PHA content (% CDM) = (Weight of PHA, g/Dried cell weight, g) × 100.

Determination of PHA functional groups and composition

The functional groups of the PHA were analyzed using Fourier-transform infrared (FTIR) spectroscopy. The PHA spectra were recorded in the wavenumber range of 650–4,000 cm^{-1} using a Perkin Elmer FTIR spectrophotometer (MA, USA). Additionally, the PHA composition was determined via the GC method, following the previously described method [9, 17].

Statistical analysis

All experiments were conducted in triplicate, and a completely randomized design was applied throughout the study. The data were analyzed using analysis of variance (ANOVA), and mean comparisons were carried out using Duncan's multiple range test. Statistical analysis was performed with the Statistical Package for Social Sciences (SPSS 24 for Windows, SPSS Inc., IL, USA).

RESULTS AND DISCUSSION

Selection of the mutant *B. thermoamylovorans* strain

Fifteen positive mutants were isolated and cultivated in PHA-producing media at 37 °C. All the bacterial isolates were designated M01–M15. However, only 1 strain (M06) of the 15 strains exhibited the ability to produce PHA with over 40% CDM (Table 1), as detected by the GC method. The M06 strain was selected for further analysis because of its high PHA production. The selection of bacterial strains with high PHA production capabilities can help overcome limitations in the large-scale economic biosynthesis process [19]. Compared with the parent strain, the mutant strain presented a significant reduction in PHA production, providing evidence for the role of this gene in PHA granule biosynthesis by *B. thermoamylovorans*. In addition, the decrease in PHA in mutants is due to several factors related to the mutation and its impact on the cell's metabolic or regulatory pathways [20].

Table 1 Production of PHA in a selected mutant of *B. thermoamylovorans* via GC analysis.

Strain	PHA production (% CDM)
Wild type	50.8 ± 1.2
Mutant M01	23.4 ± 0.6
Mutant M02	22.3 ± 1.0
Mutant M03	30.3 ± 1.3
Mutant M04	32.1 ± 1.2
Mutant M05	25.4 ± 1.0
Mutant M06	41.4 ± 1.0
Mutant M07	28.3 ± 1.5
Mutant M08	31.1 ± 1.0
Mutant M09	29.0 ± 0.9
Mutant M10	30.2 ± 1.0
Mutant M11	28.3 ± 1.3
Mutant M12	31.2 ± 1.0
Mutant M13	33.4 ± 1.0
Mutant M14	29.2 ± 1.0
Mutant M15	34.4 ± 1.1

The *fad*B and *fad*A genes are involved in fatty acid degradation, especially in the β-oxidation pathway. This pathway is crucial for breaking down fatty acids into acetyl-CoA, a central precursor for various biosynthesis processes, including PHA synthesis. When the *fad*B and *fad*A genes are deleted, fatty acid degradation is impaired, leading to a shortage of acetyl-CoA. Since acetyl-CoA is essential for the synthesis of PHA, a decreased supply limits PHA production. The β-oxidation pathway also contributes to the generation of NADH, which can be converted to NADPH through cellular metabolism. NADPH is crucial for anabolic processes, including PHA biosynthesis. Therefore, disrupting β-oxidation affects NAD(P)H levels, potentially limiting the reducing power required for PHA accumulation [21].

Moreover, deleting *fad*B and *fad*A alters the metabolic flux of carbon through the cell, rerouting it away from PHA synthesis. This effect can lead to carbon being directed toward other pathways, reducing the pool of building blocks needed for PHA production. In some bacteria, the genes involved in fatty acid metabolism are closely regulated by those involved in the PHA biosynthesis pathway. Therefore, changes in one pathway can signal shifts in regulatory networks, resulting in reduced PHA gene expression or activity [20, 21]. A decrease in PHA production in the *i-phaZ* gene of the *Acidovorax* sp. A1169 deletion mutant has also been reported [21]. Biomass and PHB accumulation were considerably lower in the

i-phaZ gene deletion mutant than in the wild-type A1169 strain when cultured under the same PHB-accumulation promotion conditions. In comparison, a concentration of 1.7 ± 0.2 g/l was achieved for the wild-type strain, and the mutant strain accumulated only 1.2 ± 0.0 g/l [21].

Production of PHA using various fatty acids

In order to improve PHA production, it is necessary to investigate and understand the growth profile of fermentable bacteria under various fermentation conditions [22]. Therefore, the potential of the wild-type and mutant strains of M06 to utilize various fatty acids was assessed. The assay was performed in shake flasks using a PHA-producing medium supplemented with sodium octanoate, decanoate or dodecanoate. Table 2 provides an overview of the tested substrates and their corresponding results. Interestingly, a similar percentage of PHA was detected in the wild-type and mutant strains. However, the mutant did not grow well in fatty acid-containing media. Higher biomass was detected in the wild-type in all the tested substrates. The highest biomass (4.5 g/l) and PHA (41.4% CDM) was detected in the wild-type strain when sodium octanoate was used as the substrate. These data suggested that the *fad*B and *fad*A genes were not crucial for the β-oxidation pathway. The *fad*B and *fad*A knockout mutants of *B. thermoamylovorans* achieved a CDM of 3.0–3.8 g/l, with a PHA content of 40.2–41.0% CDM. Additionally, the growth of the mutant strain was slightly slower than that of the parent strain, reaching the stationary phase at 60 h (Fig. 1). This result may be due to the disruption of a key gene in the β-oxidation pathway. The cell growth rate was decreased when an important gene was knockout. When *fad*B and *fad*A were deleted, fatty acid degradation via β-oxidation slowed down. The reduction of cell ability to use fatty acids or other substrates for energy and biomass synthesis was detected [10–13].

The mutant strain, however, synthesized medium-chain-length monomers, suggesting that the deletion of the *fad*B and *fad*A genes did not entirely disrupt β-oxidation. If β-oxidation had been completely inhibited, the resulting PHA would consist solely of HDD monomers. Nevertheless, it can be inferred that β-oxidation was substantially impaired, leading to greater carbon flux being directed toward PHA accumulation instead of supporting cell growth. Consequently, the PHA produced by the mutant contained a greater proportion of long-chain-length monomers compared with that produced by the wild-type (Table 2). This result was similar to that of Ouyang et al [11]. High percentages of hydroxyoctanoate (HO), hydroxydecanoate (HD) and HDD were detected in the mutant strain. A similar trend was also observed for all the tested substrates. Hydroxyhexanoate (HHx) and HO were measured when sodium octanoate was used. The HHx, HO and HD were observed when dodecanoate was utilized. However, HHx, HO, HD and HDD were detected when dodecanoate was employed (Table 2). Across all the tested substrates, the HHx fraction in the mutant strain was significantly lower than that in the parent strain. These findings indicate that the deletion of the *fad*B and *fad*A genes impaired the β-oxidation pathway.

Interestingly, the production of even-numbered PHA monomers was obtained from sodium octanoate, decanoate, and dodecanoate. This result may be due to the β-oxidation pathway within the bacteria. This pathway breaks down fatty acids into acetyl-CoA units, which are then used to synthesize PHA. The breakdown process naturally results in even-numbered carbon chains. In addition, the formation of HHx was also observed during β-oxidation. As the chain shortened, 3-HHx-CoA intermediates were accumulated and diverted into PHA biosynthesis by PHA synthase (*pha*C). Therefore, the accumulation of monomers with lower numbers than the carbon source is possible. HDD were also accumulated in wild-type and mutant strains using dodecanoate as substrate. However, a higher mol% of HDD was observed in the mutant strain. The results indicated the disruption of β-oxidation when *fad*B and *fad*A were deleted. The mutation may enhance the enzymes involved in β-oxidation, allowing for more incorporation of hydroxyacyl-CoA intermediates into the PHA chain, leading to the formation of more HDD monomers than the original strain. Moreover, mutants with defective *fad*B and *fad*A slow down β-oxidation pathway, causing longer chain hydroxyacyl-CoA intermediates such as HDD to accumulate and be polymerized into PHA [10–13].

PHA can be divided into three main groups: scl-PHA, mcl-PHA and lcl-PHA. The scl-PHA containing monomer units with 3–5 carbon atoms is brittle and a stiff material with a high degree of crystallinity in the range of 60–80%, while the mcl-PHA contains monomer units with 6–14 carbon atoms and lcl-PHA contains monomer units with > 15 carbon atoms. The mcl- and lcl-PHA are more flexible as elastic materials with low crystallinity (25%), low tensile strength, high elongation to break, and low melting temperature [23]. Table 2 indicated that only mcl monomers, including 6 (HHx), 8 (HO), 10 (HD) and 12 (HDD) carbon atoms, were accumulated while sodium octanoate, decanoate and dodecanoate were provided as substrates. Therefore, the type of monomer produced depends heavily on the substrate supplied [9]. Moreover, the utilization of different fatty acids may influence the production process. Short-chain fatty acids typically enter central metabolism (acetyl-CoA, propionyl-CoA), leading to scl-PHA monomers via *de novo* fatty acid synthesis, while medium-chain fatty acids are processed via β-oxidation. During this process, intermediates such as HHx, HO, HD and HDD can be captured by PHA synthase to produce mcl-PHA. Long-chain fatty acids enter β-oxidation more slowly. On the point

Table 2 Biomass and PHA production by *B. thermoamylovorans* parent and mutant strains when various fatty acids are used.

Substrate	Biomass (g/l)		PHA (%CDM)		Monomer composition							
					HHx		HO		HD		HDD	
	Wild type	Mutant	Wild type	Mutant	Wild type	Mutant	Wild type	Mutant	Wild type	Mutant	Wild type	Mutant
Sodium octanoate	4.5 ± 0.3	3.8 ± 0.2	41.4 ± 1.0	41.0 ± 1.0	10.0	8.2	90.0	91.8	0.0	0.0	0.0	0.0
Decanoate	4.3 ± 0.1	3.0 ± 0.1	41.1 ± 1.0	40.2 ± 1.3	15.1	12.1	70.7	73.7	14.2	20.3	0.0	0.0
Dodecanoate	4.3 ± 0.1	3.4 ± 0.2	41.0 ± 2.0	40.6 ± 1.0	12.0	4.3	58.0	63.0	19.8	20.3	10.2	12.4

HHx = Hydroxyhexanoate; HO = Hydroxyoctanoate; HD = Hydroxydecanoate; HDD = Hydroxydodecanoate.

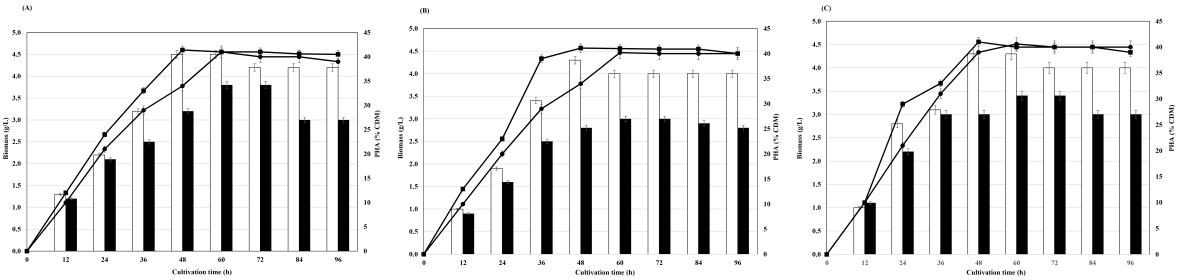

Fig. 1 Cell biomass of *B. thermoamylovorans* wild-type (□, bar) and mutant (■, bar) strains and PHA production in the wild-type (■, line) and mutant (●, line) strains in the presence of sodium octanoate (A), decanoate (B) and dodecanoate (C).

of growth rate and substrate uptake, medium-chain fatty acids generally support faster PHA production rates and higher monomer diversity, while long-chain fatty acids may slow/inhibit growth but produce long-chain monomers. Koffi et al [24] reported that the use of medium-chain fatty acids offers the best balance between high product yield and process productivity. Moreover, the substrate affects not just upstream fermentation but also the PHA produced from medium- and long-chain fatty acids, which often have lower crystallinity and are elastomeric, thereby easing and reducing the downstream cost of PHA recovery and purification [25].

Production of PHA in a 3-l fermenter using WCO as a substrate

The production of cell biomass and PHA was initially assessed in a 250 ml shaken flask using a WCO medium. *B. thermoamylovorans* was grown under optimal conditions, containing 2 g/l WCO, a temperature of 37 °C, pH 7 and agitation at 150 rpm, with cultivation halted at 48 h, following the method outlined by Sangkharak et al [9]. High CDM (3.9 ± 0.4 g/l) and PHA (40.4 ± 1.0% CDM) production were observed in the parent strain. A lower CDM (3.0 ± 0.5 g/l) with a similar content of PHA (40.3 ± 1.0% CDM) was observed in the mutant. The cultivation was subsequently scaled up via a 3-l fermenter. The kinetic parameters of the parent and wild-type strains were analyzed and are presented in Table 3.

Table 3 indicates the different production times between wild type and mutant strain. The wild type took only 48 h to enter maximum cell and PHA production while mutant required longer time (60 h). The result was similar to the previous pattern observed in Fig. 1. As previously described, the deletion of *fad*B and *fad*A mainly affected β-oxidation. WCO is mainly composed of triglycerides and free fatty acids (FFAs), which *B. thermoamylovorans* metabolizes via β-oxidation pathway to generate energy, biomass, and precursors for PHA synthesis. Without *fad*B and *fad*A, the cells cannot complete β-oxidation effectively. This effect blocks the conversion of fatty acyl-CoA to acetyl-CoA, which disrupts energy (ATP, NADH, $FADH_2$) generation from fatty acids. This eventually limits the availability of carbon precursors for cell growth and accumulates fatty acid intermediates inside the cells,

Table 3 The biomass, PHA production and kinetic parameters of the *B. thermoamylovorans* wild type and mutant strains when WCO-containing medium was used in 3-l fermentation.

Parameter	Unit	*B. thermoamylovorans*	
		Wild type	Mutant
Cultivation time	h	48	60
Biomass (CDM)	g/l	3.8 ± 0.2	3.0 ± 0.1
Consumed oil	g/l	1.3 ± 0.1	1.0 ± 0.1
PHA	g/l	1.6 ± 0.2	1.2 ± 0.1
PHA content	% CDM	42.1	40.0
μ	h^{-1}	0.08	0.05
$Y_{p/x}$	g/g	0.4	0.4
$Y_{p/s}$	g/g	1.2	1.2
$Y_{x/s}$	g/g	2.9	3.0
R_{xm}	g/l.h	0.08	0.05
R_m	g/l.h	0.03	0.02

which can be toxic or disruptive to membrane integrity and metabolism [10–13]. Therefore, wild-type strain presented high values for μ, biomass productivity, and R_m. The observed μ values were 0.08 h^{-1} for the wild-type and 0.05 h^{-1} for the mutant. The specific growth rate is a critical parameter to monitor during the process as it reflects the behaviour of the microorganisms.

Moreover, μ serves as an indicator of substrate availability, indicating that adequate amounts of substrate are present [26]. A high μ value is often associated with a high $Y_{p/x}$, which reflects the efficiency of the cells in accumulating PHA. Similar $Y_{p/x}$ values (0.4 g/g) were detected for both strains. However, a greater amount of PHA (42.1% CDM) was detected in the wild-type strain. Therefore, deleting *fad*B and *fad*A has no negative effect on biomass or PHA production. Similar values of $Y_{p/s}$ and $Y_{x/s}$ were also obtained for both strains. The $Y_{p/s}$ and $Y_{x/s}$ values mean that for each gram of substrate consumed, both strains produce the same amount of product and cell, respectively. Therefore, the $Y_{p/s}$ and $Y_{x/s}$ values confirmed that the mutation did not significantly affect the product and cell formation pathway relative to substrate consumption. This result suggests that the rate-limiting step remains unchanged, metabolic flux is conserved, and the mutation may not affect growth or product yield.

Characterization of PHA in WCO-containing medium

Extracted PHA has slightly brownish color with a rubber-like texture. The functional groups of the isolated polymers were analyzed using FTIR (Fig. 2). Typically, the detection of ester, methylene, and hydroxyl groups in the FTIR spectrum confirms the presence of PHA in the samples [27]. In this study, the FTIR spectrum displayed characteristic ester peaks at 1650 cm^{-1} (C═O) and 1050 cm^{-1} (C—O). Prominent peaks corresponding to the C—H stretching vibrations of methyl and methylene groups in alkanes were identified in the 2800–3000 cm^{-1} range. Additionally, a broad O—H stretching peak was observed at 3300 cm^{-1}. Compared with commercial polyhydroxybutyrate (PHB) and polyhydroxybutyrate-co-polyhydroxyvalerate (PHBV), the isolated polymer exhibited similar spectral features. Based on these results, the isolated polymer was confirmed to be PHA, with comparable functional groups detected in both the wild-type and mutant strains.

Moreover, PHA monomer was subjected to GC-MS analysis to confirm that the observed PHA is not merely lipid uptake from the external carbon source. PHA granule yielded methyl ester of 3-hydroxyalkanoic acid when extracted with chloroform and reacted via methanolysis. While intact or unmetabolized fatty acids would not show up as methyl ester [28]. In addition, GC-MS analysis showed the composition of PHA. The PHA composition produced by the mutant strain revealed the presence of 3-hydroxytetradecanoate (3HTD), 3-hydroxyhexadecanoate (3HHD) and 3HOD with retention times of 8.73, 9.89 and 11.97 min, respectively (Table 4). When cultivated on a medium containing WCO, the PHA consisted of 3HTD, 3HHD and 3HOD at molar proportions of 30.5, 25.2 and 44.3%, respectively. These results suggest that *B. thermoamylovorans* synthesized mcl-co-lcl PHA from the WCO substrate, which was supported by the FFA composition in WCO. Palmitic (C16:0) and oleic acid (C18:1), the predominant FFAs in WCO, are key substrates for mcl-co-lcl PHA production [29]. The strain utilized β-oxidation for PHA synthesis, which aligns with the finding of Rakkan et al [30], who reported similar PHA accumulation by *Enterobacter* sp. TS1L. However, a higher molar fraction of 3HTD was observed when WCO was used, and the production of mcl-co-lcl PHA with such a high 3HTD content has not been previously reported.

Table 4 Monomer composition (mol %) of PHA in the wild-type and mutant strains of *B. thermoamylovoans* inoculated on WCO-containing media.

Monomer	*B. thermoamylovorans*	
	Wild type	Mutant
3-hydroxybutyrate (3HB)	85	0
3-hydroxyvalerate (3HV)	15	0
3-hydroxytetradecanoate (3HTD)	0	30.5
3-hydroxyhexadecanoate (3HHD)	0	25.2
3-hydroxyoctadecanoate (3HOD)	0	44.3

The polymer isolated from the wild-type strain was identified as P(3HB-co-3HV), which contains 85 mol% 3-HB and 15 mol% 3-HV, as determined by GC-MS. Interestingly, the properties of this copolymer were comparable to those of commercial P(3HB-co-3HV). The production of different PHA monomers in mutant strains can be attributed to changes in precursor molecule availability and metabolic pathway shifts [31]. Disruption of the β-oxidation pathway can alter the types and ratios of substrates used for PHA synthesis, leading to the incorporation of various monomers (Fig. 3). The synthesis of precursors such as acetyl-CoA, propionyl-CoA, or other CoA derivatives may result in the synthesis of diverse PHA types [10]. Furthermore, when β-oxidation is inhibited, carbon sources may accumulate as medium-chain fatty acids, which can act as monomers for mcl-PHA instead of scl-PHA [12]. These findings indicate that the mutant strain of *B. thermoamylovorans* strongly grew on WCO and efficiently produced mcl-co-lcl PHA (Table 4). This finding highlights the economic potential of using WCO for mcl-co-lcl PHA production. Owing to their exceptional properties and wide-ranging applications, PHA-, especially mcl-PHA and lcl-PHA-, has garnered significant attention as a sustainable alternative to petroleum-based plastics.

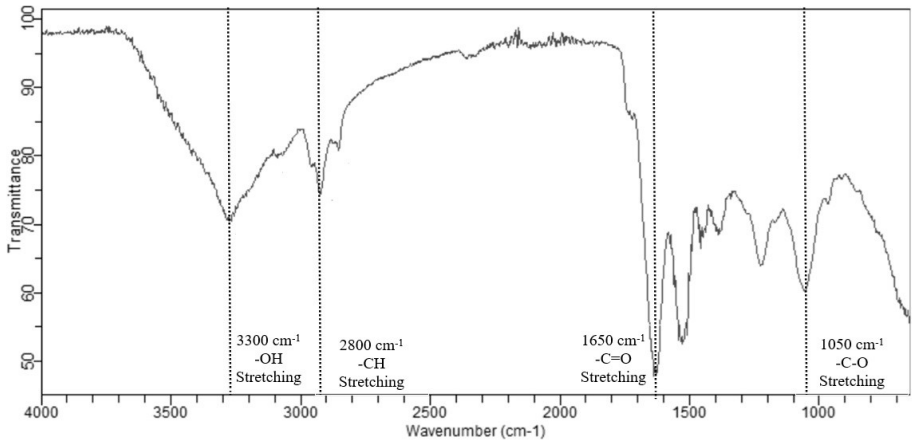

Fig. 2 FTIR spectra of an isolated polymer from a mutant strain of *B. thermoamylovoans* inoculated on a WCO-containing medium.

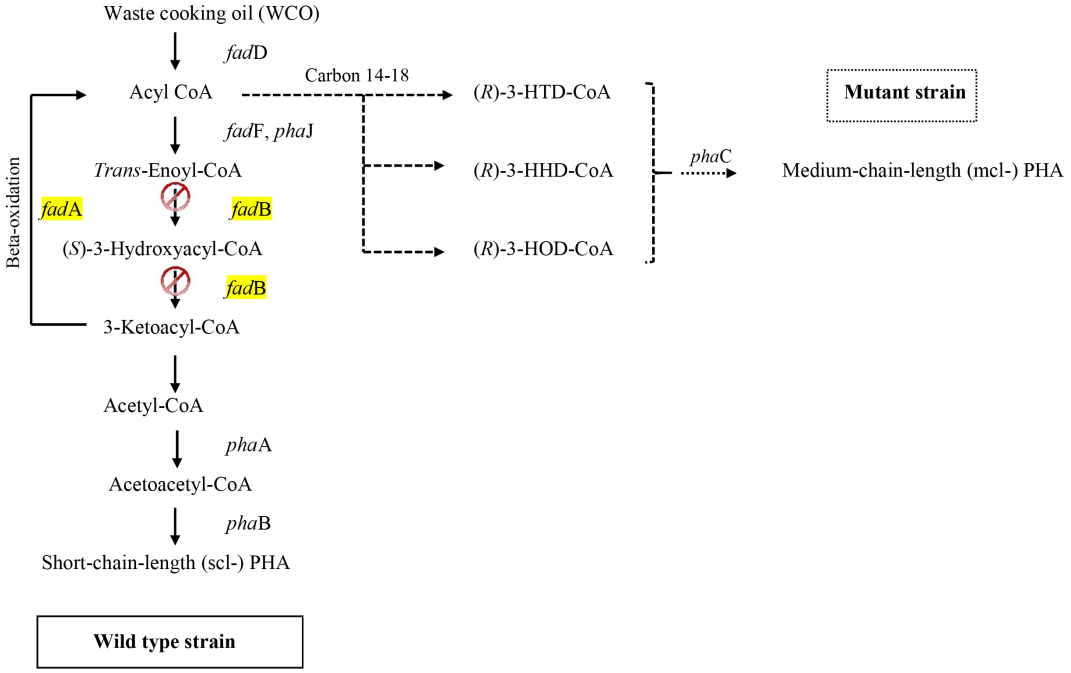

Fig. 3 A PHA biosynthesis pathway diagram, including the β-oxidation pathway, highlighting the *fad*B and *fad*A genes and indicating the putative flow of waste cooking oil consumption and PHA production in wild-type (-) and mutant strains (- -). HTD = Hydroxytetradecanoate; HHD = Hydroxyhexadecanoate; HOD = Hydroxyoctadecanoate.

CONCLUSION

A simple gene knockout method was developed for *B. thermoamylovorans*. The results revealed that, compared with other potential genes, *fad*B and *fad*A played a more significant role in fatty acid degradation. However, deleting *fad*B and *fad*A did not completely inhibit the β-oxidation pathway. PHA accumulation in the β-oxidation pathway weakened the mutant strain, which produced greater amounts of monomers with medium-co-long chain lengths. High PHA production was observed in the wild-type strain in batches and 3-l fermenters when WCO was used as the substrate. Despite these challenges, the efficient creation of PHA via the β-oxidation knockout method has significant potential to help dramatically reduce costs and create novel PHAs. Interestingly, mcl-PHA was observed by the mutant strain which has potential future applications in various fields, such as biomedical materials, flexible packaging, agricultural film, adhesives and elastomers, biolubricants, and 3D-printing and soft robotic. However, low production of mcl-PHA was

observed in the present study. Therefore, studies on the metabolic pathways affecting PHA accumulation via the knockout method should be conducted in more detail. In addition, the creation of knockout libraries and the systematic inactivation of each gene involved in PHA biosynthesis in *B. thermoamylovorans*, along with an analysis of the impact on PHA yield and quality, should be examined in future studies.

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