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Polyhydroxyalkanoate Synthesis by Recombinant *Escherichia coli* JM109 Expressing PHA Biosynthesis Genes from *Comamonas* sp. EB172

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Abstract

Recombinant *Escherichia coli* JM109 harbouring the polyhydroxyalkanoate (PHA) biosynthesis gene (phaCABco) of *Comamonas* sp. EB172, an acid tolerant microbe, was examined for the production of PHAs from various carbon sources. The study demonstrated that the recombinant *E. coli* JM109 had the potential to utilize both sugar- and acid-based carbon sources, for the biosynthesis of both poly(3-hydroxybutyrate) P(3HB) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) P(3HB-co-3HV) copolymers. In the shake flask experiments, the strain was capable of producing P (3HB-co-3HV) copolymer from mixed organic acids, and higher productivities were obtained using glucose compared to mixed acids. However, PHA accumulation was found to be similar, regardless of the carbon source used. Nitrogen supplementation in the medium was found to improve the cell dry weight, but negatively affected the 3HV formation in copolymer production. Maximum 3HV monomer (3 mol%) was obtained with C/N 42.1, using mixed acids as the carbon source. In the 2L bioreactor, the productivity and yield based on substrate utilization coefficient were found to be 0.16 g PHA/(L.h) and 0.41 g PHA/g substrate under C/N around 75, using 20 g/L glucose and 0.5 g/L ammonium sulphate, respectively. The polymer produced by the recombinant strain had molecular weight in the range of 8.5 x 105 to 1.4 x 106 Da. Overall, the ability of the recombinant *E. coli* JM109 to utilize both glucose and mixed acids, has widened its substrate selection for fermentation, including the opportunity to use renewable biomass.

Keywords: Recombinant; PhaCABco; Mixed acids; Polyhydroxyalkanoate; *Comamonas* sp. EB172

Introduction

Polyhydroxyalkanoates (PHAs) are energy storage, hydrophobic granules that can be accumulated by many microorganisms [1-4]. PHAs are biodegradable, biocompatible thermoplastics, and hence, these biopolyesters are not only the potential alternative candidates for recalcitrant synthetic plastics, but also present long-term benefits for environmental pollution issues. However, the high cost for PHA production compared to the availability of low-cost petroleum-based plastic, is the major obstacle to commercialize these biosynthesized PHAs [5,6]. The most significant factor for the high production cost of PHAs is the fermentation process, which is mainly due to the cost of raw material as well as the recovery process [7]. A great deal of effort has been made to reduce the production cost by employing superb microbial strains, as well as, developing fermentation and recovery process with cheap carbon sources and non-halogenated solvents, respectively [7-9].

Economic biotechnological PHA formation largely depends on the choice of productive microorganisms and their culture condition. Recently, in our continuous effort of utilizing mixed organic acids derived from palm oil mill effluent (POME) for PHA production, we have produced several reports on the isolation, biosynthesis and characterization of both P(3HB) and P(3HB-co-3HV) by a local, acid tolerant strain of *Comamonas* sp. designated as *Comamonas* sp. EB172 [10-14]. While developing a suitable fermentation strategy to feed mixed acids in its original form (keeping identical ratio as obtained), our focus was also to develop a non-halogenated PHA recovery system, at the end of fed-batch fermentation [10,15,16]. However, *Comamonas* sp. EB172 is known to utilize only fatty acids but not glucose or fructose [13]. Therefore, the three genes involved in the biosynthesis of PHAs by *Comamonas* sp. EB172 were cloned and characterized [17]. Meanwhile, the PHA biosynthesis genes of *Comamonas* sp. EB172 had also been cloned and heterologously expressed for its functionality, to demonstrate the ability of the isolated biosynthesis gene on PHA production in *E. coli* JM109 host.

Recombinant *E. coli* has commonly been employed for PHA production due to its convenience for genetic manipulation, fast growth, high cell density cultivation and ability to utilize inexpensive carbon sources. The strain has been reported to produce short-chain-length (scl) polyesters containing C4 or C5 monomers, such as P (3HB), poly(3-hydroxyvalerate) P(3HV), poly(4-hydroxybutyrate) P(4HB) homopolymer, or the P(3HB-co-3HV) copolymer [18] and P(3HB-co-4HB) [19]. Nevertheless, recombinant E. coli, containing the phaC1 gene from *Pseudomonas aeruginosa*, was able to produce medium-chain- length (mcl) PHAs, having C6 to C14 monomers including homopolymers of 3-hydroxyhexanoate (3HHx), 3-hydroxydodecanoate (3HDD) and terpolymer poly(3-hydroxybutyrate-co-3-

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hydroxyvalerate-co-hydroxyhexanoate), P(3HB-co-3HV-co-3HHx) [20,21], when beta-oxidation gene fadB was deleted [20]. Meanwhile, hybrid polymers containing both scl- and mcl-monomer units such as poly(3-hydroxybutyrate-co-hydroxyhexanoate), P(3HB-co-3HHx), produced naturally by *Aeromonas* caviae, can also be synthesized in recombinant *E. coli* through genetic manipulation [22,23]. For commercial PHA production, recombinant *E. coli* has also been employed by Metabolix, USA and Jiang Su Nan Tian Co. Ltd, China [24]. Metabolically engineered *E. coli* can easily express the enzyme involved in PHA biosynthesis, with various monomer compositions. Therefore, *E. coli* JM109 was chosen to express the PHA biosynthesis genes from *Comamonas* sp. EB172. The potentiality of this recombinant strain was examined in the shake flasks, and bioreactor studies using both refined and non-refined carbon sources.

In this study, the performance of recombinant *E. coli* JM109 was examined for P (3HB-co-3HV) copolymer production, and better cell growth using glucose and mixed organic acids as the carbon sources. The polymer produced was further characterized for its chemical structure and molecular weight. The data reported herewith, may be useful for future work using recombinant strain, with local renewable substrates such as POME and oil palm fronds (OPF) [25].

Materials and Methods

Bacterial strains, plasmids and culture conditions

Recombinant *E. coli* JM109 that harboured plasmid pGEM'-phaCABCo containing PHA biosynthesis gene, phaCABCo of *Comamonas* sp. EB172, was used in this study [17]. *E. coli* JM109 is a general host for cloning. Through this study, we can observe the substrate or enzyme ability of the cloned genes for the PHA accumulation under various conditions. Hence, simple *E. coli* bacteria strain is necessary rather than the expression host such as *E. coli* DH5α. The *E. coli* DH5α can be the strain for future genetic modification and process optimization. Recombinant *E. coli* JM109 was cultivated at 37°C and 200 rpm in the Luria-Bertani medium, comprising the following components: 10 g casein peptone, 10 g sodium chloride and 5 g yeast extract in 1 L of distilled water. The antibiotic ampicillin (50 μg/mL) was added to the culture medium, to maintain the stability of the plasmids.

PHA accumulation in shake flask

The recombinant *E. coli* JM109 was pre-cultured in the Luria-Bertani medium at 37°C and 200 rpm for 14 h, and 10% (v/v) of the culture (OD600nm≈3.0) was inoculated into a 250 mL shake flask, containing 50 mL of nitrogen-limited mineral salts (MS) medium (pH 7.0). The MS medium consisted of (in g/L) 5 g KH_2PO_4, 2 g K_2HPO_4, 1 g $(NH_4)_2SO_4$, 0.4 g $MgSO_4{\cdot}7H_2O$ and 1 mL of trace element solution [17]. Ampicillin (50 μg/mL) was added, when it was necessary for plasmid maintenance. Glucose was sterilized separately and was added to the rest of the medium, before inoculation to avoid caramelization effect. The mixtures of three acids (acetic: propionic: n-butyric acid) in a ratio of 3:1:1, were sterilized along with other salts in the MS medium, to give a final concentration of 10 g/L. The mass ratio of 3:1:1 for the mixed organic acids was to simulate anaerobically the fermented POME, as the carbon source [26]. The cultures were cultivated at 37°C and 200 rpm for 48 h in the incubator shaker. The cell growth, cell concentration and final pH were monitored during the fermentation.

PHA accumulation in 2 L bioreactor

The pre-cultured recombinant *E. coli* JM109 in a 100-mL Luria-Bertani medium, in a 500-mL shake flask was used as the seed culture. The seed culture was incubated at 37°C and agitated at 200 rpm for 14 h. One hundred millilitres (10% v/v) of the seed culture (OD600nm≈ 3.0) was inoculated into a 2 L bioreactor, with a 900-mL MS medium supplemented with 10 g/L, 20 g/L glucose or 10 g/L mixed acids as the carbon source. Three different concentrations of ammonium sulphate (without nitrogen addition, 0.5 g/L and 1.0 g/L) were supplemented in the MS medium. The cells were grown at 37°C and pH 7 (controlled using 1 M NaOH) throughout the experiment. The dissolved oxygen tension (DOT) level was maintained above 30% of air saturation, by automatically raising the agitation speed from 150 to 600 rpm. The air flow rate was kept at 1 vvm throughout the fermentation. The batch fermentation was cultivated for 24 h, depending on the exhaustion of the carbon sources in the MS broth. The cell growth, cell concentration and PHA accumulation were monitored during the course of the fermentation.

Gas chromatography analysis

Grown cells were harvested by centrifugation (6000 x g, 10 minutes, 4°C), washed with distilled water and lyophilised. The lyophilized cells after methanolysis at 100°C for 140 minutes, in the presence of sulphuric acid and methanol (15:85 v/v) [27], were subjected for cellular PHA content and polymer composition by gas chromatography (Shimadzu, GC2014) analysis.

Extraction and purification of polymer from cell

Polymers were extracted from the lyophilized cells using the modified method of Amirul et al. [28]. One gram of lyophilized cells was stirred overnight in 250 mL of chloroform at room temperature. The mixture of cell debris and polymers was separated through Whatman No. 1 membrane filter paper, with the pore size of 11 μm. PHA dissolved in chloroform and was recovered by precipitating using cold methanol, in the ratio of 1:5. The precipitated polymer was filtered using 0.45 μm PTFE membrane filter and dried overnight at room temperature. The extracted pure polymer was then characterized for its chemical structure and molecular weight, by proton nuclear magnetic resonance (1HNMR, 500 MHz JEOL JNM-ECP500 FT NMR) and size-exclusion chromatography (SEC, TOSOH HLC-8120), respectively [29].

Characterization of polymer

1H NMR spectra were recorded on a 500 MHz JEOL JNM-ECP500 FT NMR. Chloroform-d (CDCl3) was used as a solvent. Chemical shifts were reported as δ values (ppm) relative to internal tetramethylsilane (TMS) in CDCl3, unless otherwise stated. The molecular weights of the polymers were measured on a TOSOH HLC-8120 SEC system with refractive index (RI) and ultraviolet (UV, λ=254 nm) detectors, under the following conditions: TSKgel Super HM-H linear column (linearity range, 1x103 - 8 x106; molecular weight exclusion limit, 4 x108), chloroform eluent at a flow rate of 0.6 mL min-1, and column temperature of 40°C. The calibration curves for SEC analysis were obtained, using polystyrene standards with a low polydispersity (5.0x102, 1.05 x103, 2.5 x103, 5.87 x 103, 9.49 x103, 1.71x104, 3.72 x104, 9.89x104, 1.89x105, 3.97x105, 7.07 x105, 1.11x106, TOSOH Corp.).

Data analysis

Statistical analysis of the experimental results was carried out using MSTAT-C software. Duncan's Multiple Range Test at 5% ($p<0.05$) alpha level was used for mean experimental data comparisons.

Results and Discussions

Effect of carbon and nitrogen source on PHA biosynthesis in shake flask

E. coli JM109 is known to be a host that can effectively express the enzyme, and capable to synthesize various PHAs with different monomer compositions without degradation [30]. The mechanism for PHA biosynthesis in *E. coli* is different from other bacteria, where nutrient limiting condition is not necessary for PHA production [31]. Thus, nitrogen source provides especially for cell growth. However, Steinbüchel and Pieper [32] reported that recombinant *A. eutrophus* is capable for P (3HB-co-3HV) copolymer accumulation, from various unrelated carbon sources, when nitrogen is limited and carbon sources are supplementing in excess. In this study, the effect of different carbon sources on cell growth and PHA production from the recombinant *E. coli* JM109 harbouring plasmid pGEM'-phaCABCo, was carried out in the shake flask. The fermentation in the shake flasks was carried out with or without nitrogen source, to examine the effect of nitrogen source on the growth and PHA accumulation of E. coli.

Table 1 shows the cell growth and PHA production of the recombinant *E. coli* JM109, using different carbon sources (i.e. glucose and mixed acids). It was observed that the recombinant *E. coli* JM109 preferred glucose compared to mixed acids, as its carbon source for growth. The cell titre showed a significant difference ($p<0.05$), depending on the initial concentration of carbon source. It can be observed that the cell growth was affected by the initial concentration of glucose, in which higher glucose concentration reduced the CDW formation. However, there was no significant difference on PHA accumulation, when a different type of carbon source was used. There was only a slight increase in PHA accumulation, once glucose was used (Table 1). This may be due to the substrate concentration, inhibitory of the initial amount of glucose used. However, the recombinant still can utilize for PHA biosynthesis. Therefore, the use of higher concentration of glucose (40 g/L), not only decreased the cell growth but also P (3HB) accumulation (data not shown).

Glucose was utilized for better cell formation and PHA accumulation compared with mixed organic acids, whereby they only affected accumulation of PHA. Nevertheless, declined growth of recombinant *E. coli* JM109 was observed, in medium containing 10 g/L mixed acids supplemented with nitrogen source. The mixed organic acids were consumed more effectively by recombinant without nitrogen supplement, compared to the cultivation under same condition with 1 g/L nitrogen source. From Table 1, it is seen that the recombinant *E. coli* JM109 was able to utilize mixed organic acids for P (3HB-co-3HV) copolymer accumulation. A similar observation was also reported recently [17]. This is in agreement with previous finding by Chien et al. [33], whereby it was discussed that the presence of propionic acid in the fermentation broth, contributed to P (3HB-co-3HV) copolymer biosynthesis [17, 33].

Table 1 and Table 2 show the effect of nitrogen on the CDW and PHA accumulation of the recombinant *E. coli* JM109. When the recombinant *E. coli* JM109 was cultivated in the MS medium containing nitrogen source [1 g/L of $(NH_4)_2SO_4$] and 10 g/L glucose, CDW increased from 1.5 g/L (Table 1, experiment 1) to 2.3 g/L (Table 2, experiment 1) and PHA content increased from 34% to 46%, respectively. Meanwhile, Lee and Chang [34] reported that addition of complex nitrogen sources in the fermentation medium, could significantly enhance the concentration of accumulated P (3HB), in both shake flasks and fed-batch fermentation of the recombinant *E. coli* harbouring the genes from *Alcaligenes eutrophus*. Once again, the CDW and PHA contents of increased amount of glucose (20 g/L) did not show any significant difference to 10 g/L glucose as the carbon source (Table 2, experiment 2).

The results showed that both CDW and PHA content were improved, when the fermentation was supplemented with $(NH_4)_2SO_4$. This indicates that PHA biosynthesis in recombinant *E. coli* happened during growth, and not under nutrient limitation like those phenomenon observed in wild type PHA producer such as *C. necator* [31]. However, the complex medium could reduce the P(3HB) accumulation while supplementation of small amount of complex nitrogen sources [34], amino acids or oleic acids [35] could improve the P(3HB) synthesised. The addition of nitrogen sources will increase the amount of acetyl-CoA and/or NADPH in the PHA metabolic pathway for PHA production [35,36]. Chien et al. [33] reported that total CDW of recombinant *E. coli* was increased as the increment of yeast extract, with glucose as carbon source in medium. The increased concentration of yeast extract also, led to improve the PHA content in the recombinant cells. Hence, the CDW and PHA content in the cells can be improved under regular carbon and nitrogen supplement in recombinant cells.

Experiment	Carbon source	Conc. g/L	Remaining carbon source (g/L)	CDW (g/L)	PHA content[II] (wt%)	PHA composition[II] (mol%)	
						3HB	3HV
Control[III]	Mixed[I] acids	10	NA	0.4 ± 0.07	ND	ND	ND
Control[IV]	Glucose[I]	10	NA	1.6 ± 0.3	46.1 ± 3.8	100	0
1	Glucose[I]	10	0.3	1.5[a] ± 0.2	34.3[a] ± 1.0	100.0	0
2		20	0.7	0.8[b] ± 0.03	34.8[a] ± 0.5	100.0	0
3	Mixed acids[I]	10	5.4	0.5[c] ± 0.01	32.0[a] ± 2.2	97.7	2.3

CDW, cell dry weight; PHA, polyhydroxyalkanoates; 3HB, (3-hydroxybutyrate); 3HV, (3-hydroxyvelerate); ND, not detected; NA, not analyzed.

Data shown are means of triplicates. Mean data accompanied by different superscript letters, are significantly different (Duncan's Multiple Range Test, $p < 0.05$).

[I] Incubated for 48 h at 37 °C at 200 rpm in MS medium, without 1 g/L $(NH_4)_2SO_4$ supplemented with 50 µg/mL of ampicillin. Sterilized glucose was added during inoculation, while mixed acids were supplied directly prior to autoclaving and pH was adjusted using 1M NaOH.

[II] PHA content in lyophilized cells was determined by GC.

[III] *Escherichia coli* JM109 was cultivated in MS medium with mixed acids as negative control.

[IV] *Escherichia coli* JM109 harbouring pGEM'-phbCAB$_{Re}$, PHA biosynthesis gene from *C. necator* was cultivated in MS medium with 10 g/L glucose as positive control.

Table 1: PHA biosynthesis from various carbon sources by recombinant *E. coli* JM109 harbouring pGEM'-phaCAB$_{Co}$ plasmid with nutrient limitation[I].

On the other hand, declined growth of the recombinant *E. coli* JM109 was observed, in the medium containing mixed acids supplemented with nitrogen source. This may be due to the inhibitory effect of mixed organic acids, in the ratio of 3:1:1 (acetic acid: propionic acid: n-butyric acid). Even with the model organism, C. necator, the maximum CDW and PHA contents using 3 g/L mixed acids in the ratio of 3:1:1 (acetic acid: propionic acid: n-butyric acid) were shown to be 1.1 g/L and 58.2 wt%, respectively, after 41 h of cultivation [37]. However, it has to be noted that the tolerance level of mixed acids differs among bacterial species, and PHA accumulation in native producers such as in C. necator, which require nitrogen limitation.

As shown in Table 2, the PHA accumulation was slightly better, when the MS medium containing mixed organic acids was supplemented with $(NH_4)_2SO_4$. It was observed that with the presence of nitrogen in the culture medium, PHA accumulation was slightly increased from 32% to 37%, but the 3HV monomer content was decreased (Table 2, experiment 3). Similar observations were reported by Kim et al. [38] in Alcaligenes sp. SH-69, in which nitrogen source was found to affect the chemical composition of polymer. Chien et al. [33] reported that the increase in concentration of yeast extract in cultivation medium had decreased the 3HV formation in P (3HB-co-3HV) copolymer produced.

In order to examine the effect of nitrogen and 3HV molar fraction of P(3HB-co-3HV) copolymer, the recombinant *E. coli* JM109 was grown in a different concentration of $(NH_4)_2SO_4$, with equal amount of mixed acids at 10 g/L in the shake flask. As shown in Table 3, more 3HV can be produced with lower supplementation of the nitrogen source. The highest 3HV fraction of 2.65 mol% was observed, with an addition of 0.5 g/L $(NH_4)_2SO_4$ (C/N ratio at 42.1) and with low CDW. A C/N ratio of more than 21 mol /mol enhanced the cell growth and PHA accumulation, whereas at lower C/N ratio, both the 3HV and CDW were decreased (Table 3). On the other hand, PHA content was the highest at 21 mol/mol C/N ratios, using mixed organic acids. Obviously, the CDW and fraction of 3HV monomer of the recombinant *E. coli* were decreased by 1 g/L $(NH_4)_2SO_4$ addition, compared to lower concentration of nitrogen addition. Hence, excess carbon sources and limiting nitrogen supplementation condition were needed for synthesizing the copolymer, and affecting the growth of the constructed recombinant *E. coli* JM109.

Batch fermentation of recombinant *E. coli* JM109 in 2 L bioreactor

Batch fermentation was performed in a 2 L bioreactor, to get higher cell and PHA concentrations under controlled condition, compared to the shake flask fermentation. As shown in Table 1 and Table 2, the availability of propionic acids in the mixed organic acids, in the medium, could lead to the biosynthesis of 3HV monomer for the accumulated polymer, while the utilisation of glucose in the medium could improve the cell growth. Moreover, the addition of nitrogen was able to increase the CDW and PHA accumulation, using glucose in the medium as substrate. Table 4 illustrates the kinetic parameter values

Experiment	Carbon source	Conc. (g/L)	Remaining carbon source (g/L)	CDW (g/L)	PHA content[II] (wt%)	PHA composition[II] (mol%)	
						3HB	3HV
Control[III]	Mixed[I] acids	10	NA	0.6 ± 0.07	ND	ND	ND
Control[IV]	Glucose[I]	10	NA	1.7 ± 0.2	48.0 ± 1.6	100	0
1	Glucose[I]	10	0.0	2.3[a] ± 0.03	46.4[a] ± 2.6	100	0
2		20	0.5	2.3[a] ± 0.06	44.1[a] ± 8.1	100	0
3	Mixed acids[I]	10	7.3	0.2[b] ± 0.01	37.7[a] ± 1.8	99.7	0.3

CDW, cell dry weight;PHA, polyhydroxyalkanoates; 3HB, (3-hydroxybutyrate); 3HV, (3-hydroxyvelerate); ND, not detected; NA, not analyzed.

Data shown are means of triplicates. Mean data accompanied by different superscript letters, are significantly different (Duncan's Multiple Range Test, $p < 0.05$).

[I]Incubated for 48 h at 37 °C at 200 rpm in MS medium containing 1 g/L $(NH_4)_2SO_4$ supplemented with 50 µg/mL of ampicillin. Sterilized glucose was added during inoculation, while mixed acids were supplied directly, prior to autoclaving and pH was adjusted using 1M NaOH.

[II] PHA content in lyophilized cells was determined by GC.

[III] *Escherichia coli* JM109 was cultivated in MS medium with mixed acids as negative control.

[IV] *Escherichia coli* JM109 harbouring pGEM'-phbCAB$_{Re}$, PHA biosynthesis gene from *C. necator* was cultivated in MS medium with 10 g/L glucose as positive control.

Table 2: PHA biosynthesis from various carbon sources by recombinant *E. coli* JM109 harbouring pGEM'-phaCAB$_{Co}$ plasmid with 1 g/L $(NH_4)_2SO_4$[I].

Experiment	$(NH_4)_2SO_4$ (g/L)	C/N	CDW (g/L)	3HV mol fraction (%)[II]
Control[III]	0.0	0	0.4 ± 0.07	ND
Control[IV]	0.0	0	0.5 ± 0.08	17.85 ± 0.49
1	0.0	0	0.45 ± 0.00[a]	2.32 ± 0.02[b]
2	0.5	42.1	0.36 ± 0.04[a]	2.65 ± 0.11[a]
3	1.0	21.0	0.24 ± 0.00[b]	0.31 ± 0.09[c]

C/N, carbon to nitrogen ratio; CDW, cell dry weight; 3HV, (3-hydroxyvelerate).

Data shown are means of triplicates. Mean data accompanied by different superscript letters, are significantly different (Duncan's Multiple Range Test, $p < 0.05$).

[I]Incubated for 48 h at 37 °C at 200 rpm in MS medium with different amount $(NH_4)_2SO_4$ supplemented with 50 µg/mL of ampicillin. Mixed acids (10 g/L) were supplied directly, prior to autoclaving and pH was adjusted using 1M NaOH.

[II] PHA content in lyophilized cells was determined by GC.

[III] *Escherichia coli* JM109 was cultivated in MS medium with mixed acids as negative control.

[IV]*Escherichia coli* JM109 harbouring pGEM'-phbCAB$_{Re}$, PHA biosynthesis gene from *C. necator* was cultivated in MS medium with 10 g/L mixed organic acids as positive control.

Table 3: The cell dry weight (g/L) and 3HV molar fraction (%) using different amount of $(NH_4)_2SO_4$ with 10 g/L mixed organic acids as carbon source[I].

for batch fermentation, using 10 and 20g/L glucose as substrate with 0.5 g/L $(NH_4)_2SO_4$ (37.7 and 75.5 C/N, respectively). The recombinant *E. coli* JM109 was cultivated in 10 g/L and 20 g/L glucose supplemented with 0.5g/L, 1g/L and without $(NH_4)_2SO_4$. However, higher productivity was observed in the medium supplemented with 0.5g/L $(NH_4)_2SO_4$. Although, the PHA yield based on PHA biosynthesis per cell (g PHA/g cell) in the fermentation using 10 and 20 g/L of glucose was similar, the substrate utilization coefficient, Yp/s (g PHA/g substrate), and productivity were higher when 20 g/L glucose was used as the sole carbon source. Bioreactor fermentation performed at controlled pH and DO can certainly improve the biomass and PHA production, as compared to shake flask fermentation with only controlled initial pH. The lower level of PHA biosynthesis using 10 g/L glucose could be due to rapid exhaustion of glucose concentration (less than 1 g/L) after 18 h cultivation. Slightly higher PHA biosynthesis per cell (g PHA/g cell) of the recombinant *E. coli* JM109 was obtained in the fermentation using 10 g/L glucose. However, the best productivity, 0.16 g PHA/ (L.h), was observed in medium with 20 g/L glucose supplemented with 0.5 g/L $(NH_4)_2SO_4$.

Figure 1 and 2 shows the profile for the OD, CDW and PHA content, for the batch reaction using 10 g/L and 20 g/L glucose with 0.5g/L $(NH_4)_2SO_4$. Higher concentration of glucose, the cell growth was slower than the PHA accumulation compared to the lower glucose concentration, where the cell growth almost proportional to the PHA accumulation. The recombinant bacteria preferred to produce carbon compound intracellularly, and later cell metabolism with lower concentration of carbon source.

On the other hand, batch fermentation was performed by feeding 10 g/L mixed organic acids with 1 g/L $(NH_4)_2SO_4$, to observe the ability of a recombinant cells that can utilise mixed organic acids derived from POME, rather than glucose as substrate. However, the experiment further proved that the mixed organic acids were not an ideal sole carbon substrate for PHA accumulation, in batch fermentation. On the contrary, when mixed organic acids were used as the carbon source, lower productivity and yield [0.003g PHA/ (L.h-1) and 0.03g PHA/g substrate, respectively] were achieved. During batch fermentation with mixed organic acids, 1.9 mol% of 3HV monomer fraction was observed at 30 h cultivation time. The 3HV monomer was decreased to 1.1 mol%

Kinetic parameter values	Glucose (g/L)		
	20[I]	10	20
t (h)	24	24	24
PHA content (%wt)	-	65.34	62.65
X_m(g cell/L)	2.65	3.03	5.9
P_m (g PHA/L)	-	2.05	3.77
μ_m (h^{-1})	0.13	0.08	0.19
$Y_{x/s}$ (g cell/g substrate)	-	0.43	0.64
$Y_{p/s}$ (g PHA /g substrate)	-	0.29	0.41
$Y_{p/x}$ (g PHA/g cell)	-	0.68	0.64
P_r (g PHA/(L.h^{-1}))	-	0.09	0.16

t, time at the maximum cell concentration (h); X_m, maximum cell concentration (g cell/L); P_m, maximum PHA concentration (g PHA/L); μ_m, maximum specific growth rate (h^{-1}); $Y_{x/s}$, growth yield coefficient (g cell/g substrate); $Y_{p/s}$, PHA yield based on the substrate utilized coefficient (g PHA /g substrate); $Y_{p/x}$, PHA biosynthesis per cell (g PHA/g cell); P_r, PHA productivity (g PHA/(L.h^{-1}).

[I] *Escherichia coli* JM109 was cultivated in MS medium with 20 g/L glucose as negative control.

Table 4: Comparison of kinetic parameter values of PHA biosynthesis in 2 L bioreactor using glucose as carbon source.

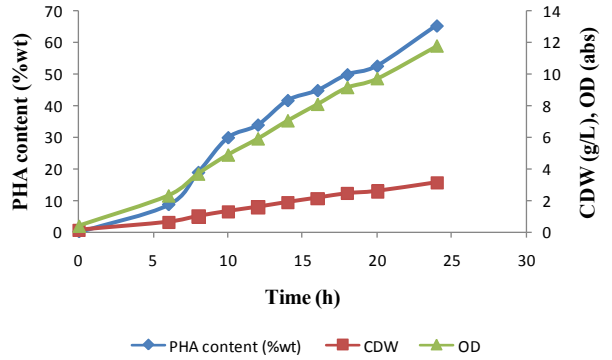

Figure 1: Profile of OD, CDW and PHA content during the batch fermentation using 10 g/ L glucose with 0.5 g/ L $(NH_4)_2SO_4$.

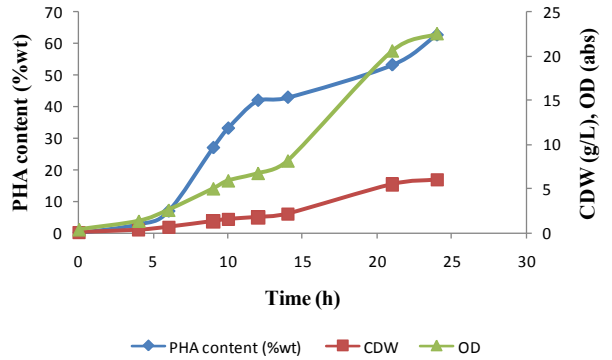

Figure 2: Profile of OD, CDW and PHA content during the batch fermentation using 20 g/L glucose with 0.5 g/L $(NH_4)_2SO_4$.

at 33 h, at the end of the fermentation with the increasing of PHA content from 22.5 to 32.2 wt%, respectively (data not shown). Table 5 shows some recombinant *E. coli* which are used for production of P(3HB-co-3HV). The mixed organic acids were mainly fed for accumulation of copolymer, and the total PHA accumulated comparable with previous study. Therefore, mixed organic acids derived from POME can be used as an alternative carbon source, as the recombinant *E. coli* JM109 can still utilize this less favourable substrate for PHA accumulation. For this purpose, a proper fermentation strategy using 2L bioreactor necessary established to produce co-polymers, using mixed organic acids by the designed recombinant.

Polymer characterization

The methyl esters of the constituent hydroxyalkanoic acids were analyzed using GC. The GC result showed that PHA synthesized from the recombinant *E. coli* JM109, contained 3HV monomer fraction. Hence, 1H NMR analysis was carried out to determine the chemical structure of the polymer. Figure 1 shows the 1H NMR spectrum of polymer produced by the recombinant *E. coli* JM109, from the 2 L bioreactor. The spectrum confirmed that the PHA synthesized by the recombinant *E. coli* JM109 in batch fermentation, using 10 g/L mixed organic acids with 1 g/L nitrogen supplementation, was a P (3HB-co-3HV) copolymer. The present signals for 3HB molar fraction were identified at 1.27 ppm, 2.4-2.6 ppm and 5.27 ppm, which represent methyl (CH3), methylene (CH2) and methine (CH) groups, respectively. The signals of 3HV monomer were attributed by the methyl signal, shown by a triplet at 0.9 ppm. Other characteristic signals

Organism	Substrate	Mode of operation	CDW (g/L)	PHA content (%wt)	Reference
Recombinant *E. coli*	Mixed organic acids	Shake flask	0.2	37.7	This study
Recombinant *E. coli*	Mixed organic acids	Batch	0.3	32.26	This study
Recombinant *E. coli*	Glucose + sodium propionate	Shake flask	3.3	37.6	[33]
Escherichia coli XL10-Gold	Glucose + propionate	Fed-batch	1.35	8.6	[39]
E. coli BW25113	Glucose	Shake flask	2.83	18.96	[40]
Recombinant *E. coli*	Glucose + propionate	Shake flask	18.0	29.5	[41]

Table 5: Production of P(3HB-*co*-3HV) using recombinant *E. coli*.

of 3HV unit cannot be seen, which could either due to low intensity or overlapping with 3HB characteristic signal, such as methylene signals at 2.4-2.6 ppm. Thus, based on the GC and 1H NMR analyses, it was confirmed that the polymer was P (3HB-co-3HV) copolymer, produced from mixed organic acids as the sole carbon source.

Table 6 shows the molecular weight and polydispersity index (PDI) of chloroform-extracted polymers produced by the recombinant *E. coli* JM109, from the 2 L bioreactor with glucose or mixed organic acids as the carbon source. Molar mass is an important factor determining physical properties of polymers, and is known to vary with substrate and culture conditions [42]. The average numbers of molecular weight (Mn) of the P (3HB) recovered from *A. eutrophus* and the recombinant *E. coli* strain, by chloroform extraction was reported to be 1.2 x 106 Da and 1.53 x 106 Da, respectively [43]. In this study, the weight average of molecular weight (Mw) of the polymer accumulated by the recombinant *E. coli* JM109 was in the range of 8.5 x 105 to 1.4 x 106 Da. The Mw of the polymer was not affected by the difference in PHA content of the cells. However, the type of carbon source and the different concentration of substrate could affect the Mw of the polymers. Under the same cultivation condition, the PHA produced from 10 g/L glucose, represented the highest Mw compared to 20 g/L glucose and 10 g/L mixed organic acids. These molecular weights were found to be higher than the PHA polymer synthesized by the recombinant E. coli, expressing a different PHA synthase [44,45]. The highest Mw was achieved from batch fermentation with 10 g/L glucose, whereas the lowest PDI was obtained from the fermentation of mixed acids (Table 6). A high molecular weight with a low polydispersity is usually desired in the production of commodity thermoplastics. A molecular weight of 6 x 105 Da or above, is considered acceptable for thermoplastic applications of scl-P(3HB-co-3HV) [46]. On the other hand, in coatings, pressure-sensitive adhesives, polymer binding agents in organic-solvent-free paints and in a range of medical applications, low molecular weights are preferable [47]. Apparently, the PDI value was found to be similar to the values reported for the PHAs synthesized by other bacteria.

Experiment	Carbon source	M_n[II] (kDa)	M_w[II] (kDa)	PDI[II] (M_w/M_n)
1	10 g/L Glucose	740	1440	1.95
2	20 g/L Glucose	510	1000	1.96
3	10 g/L Mixed acids	570	850	1.50

CDW, cell dry weight; PHA, polyhydroxyalkanoates; 3HB, (3-hydroxybutyrate); 3HV, (3-hydroxyvelerate), M_n, number average of molecular weight; M_w, weight average of molecular weight; PDI, polydispersity index.

[I] cultivated at 37°C, pH 7 and DO level was maintained at 30% in MS medium containing 1 g/L $(NH_4)_2SO_4$ supplemented with 50 µg/mL of ampicillin for 24 h. The air flow was kept at 1 vvm. Sterilized glucose was added during inoculation while mixed acids were supplied directly, prior to autoclaving and pH was adjusted using 1M NaOH.

[II] Determined by GPC analysis

Table 6: Molecular weight and polydispersity of PHA produced by recombinant *E. coli* JM109 with 1 g/L $(NH_4)_2SO_4$ in 2 L bioreactor[I].

Conclusion

Unlike the wild type *Comamonas* sp. EB172, the recombinant *E. coli* JM109 was able to utilize both glucose and mixed organic acids, to produce P (3HB-co-3HV) copolymer. This implies that recombinant *E. coli* JM109 has potential to utilize the waste-stream by-products from oil palm industry, such as mixed acids from POME and/or mixed sugars from oil palm frond juice, for PHA production. From the shake flask and batch fermentation, we know that P (3HB) can be accumulated using glucose and mixed organic acids. However, P (3HB-co-3HV) copolymer only accumulated, using mixed organic acids. However, there are two main issues a) the low cell growth and b) production of co-polymer from supplied carbon source. Therefore, development of a fermentation strategy that is capable to cater the issues is necessary. PHA was accumulated intracellularly, therefore, increasing the CDW will basically improve the PHA content. A proper feeding strategy can be suggested, for employing as suitable mode for PHA production with mixed organic acids. Fed-batch cultivation is an industrial preferred mode of operation, in order to achieve high cell density and reduce the substrate inhibitory. Thus, the mixed organic acids concentration can be maintained for biological and metabolic activities, at appropriate level. Fed-batch fermentation may improve CDW, PHA accumulation or 3HV monomer fraction. On the other hand, genetic modification on threonine pathway or protein engineering on PHA biosynthesis genes, may improve the substrate or enzyme specificity will also be our future focus. The polymer produced from this study has desired molecular weight, suitable for thermoplastic applications. Based on the properties, co-polymer will be preferred in PHA production since it is more valuable in applications.

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