Microbial production of medium-chain-length 3-hydroxyalkanoic acids by recombinant Pseudomonas putida KT2442 harboring genes fadL, fadD and phaZ

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Abstract

Monomers of microbial polyhydroxyalkanoates, mainly 3-hydroxyhexanoic acid (3HHx) and 3-hydroxyoctanoic acid (3HO), were produced by overexpressing polyhydroxyalkanoates depolymerase gene phaZ, together with putative long-chain fatty acid transport protein fadL of Pseudomonas putida KT2442 and acyl-CoA synthetase (fadD) of Escherichia coli MG1655 in P. putida KT2442. FadLPp, which is responsible for free fatty acid transportation from the extracellular environment to the cytoplasm, and FadDEc, which activates fatty acid to acyl-CoA, jointly reinforce the fatty acid β-oxidation pathway. Pseudomonas putida KT2442 (pYZPst01) harboring polyhydroxyalkanoates depolymerase gene phaZ of Pseudomonas stutzeri 1317 produced 1.37 g L−1 extracellular 3HHx and 3HO in shake flask studies after 48 h in the presence of sodium octanoate as a sole carbon source, while P. putida KT2442 (pYZPst06) harboring phaZPst, fadDEc and fadLPp achieved 2.32 g L−1 extracellular 3HHx and 3HO monomer production under the same conditions. In a 48-h fed-batch fermentation process conducted in a 6-L fermentor with 3 L sodium octanoate mineral medium, 5.8 g L−1 extracellular 3HHx and 3HO were obtained in the fermentation broth. This is the first time that medium-chain-length 3-hydroxyalkanoic acids (mcl-3HA) were produced using fadLPp and fadDEc genes combined with the polyhydroxyalkanoates depolymerase gene phaZ.

Introduction

Polyhydroxyalkanoates are storage compounds deposited as insoluble inclusion bodies in many bacteria (Steinbüchel & Füchtenbusch, 1998). Some polyhydroxyalkanoates have various thermal and mechanical properties that are attractive in both medical devices and tissue engineering (Tian et al., 2001; Chen & Wu, 2005a). As monomers of polyhydroxyalkanoates, hydroxyalkanoic acids (HA) are R-enantiomerically pure chemicals (Ren et al., 2005). So far, over 150 types of polyhydroxyalkanoates monomers have been reported (Chen & Wu, 2005b), providing abundant chiral building blocks for synthesis of fine chemicals such as antibiotics, vitamins, aromatics and pheromones (Chiba & Nakai, 1985; Lee et al., 1999). Among these HA, 3-hydroxybutyric acid (3HB) was investigated for its in vivo physiological functions (Cheng et al., 2006). Takahashi et al. (1994) showed that medium-chain-length polyhydroxyalkanoates monomers have a potential pharmaceutical value. Another study indicated that R-3-hydroxy-n-phenylalkanoic acids have antimicrobial activity against food pathogens such as Listeria monocytogenes (Sandoval et al., 2005). Pure aliphatic R-3-hydroxyoctanoic acid also displayed antibacterial activity (Ruth et al., 2007).

Early in 1964, in vivo biodegradation of PHB was reported to obtain 3HB (Merrick & Doudoroff, 1964). Morikawa & Marchessault (1981) used a polyhydroxyalkanoates pyrolysis process and obtained 60% crotonic acid from purified PHB. Other methods, including chemical degradation, methyl esterification, hydrolytic degradation, enzymatic degradation and chemical synthesis were also used to produce HA. High purification costs and application
difficulties still restrict HA industrial production (de Roo et al., 2002).

Microbial production of chiral HA has been considered to be sustainable environmentally and economically. Liu et al. (2007) produced 12 g L\(^{-1}\) 3HB using a one-stage fermentation process in a 6-L fermentor grown with *Escherichia coli* BW25113 harboring *phaA, phaB* and *tesB*. Zheng et al. (2004) produced a 1 g L\(^{-1}\) 3-hydroxydecanoic acid (3HD) monomer in *E. coli* harboring *phaG* and *tes* genes from a fatty acid *de novo* synthesis pathway.

Polyhydroxyalkanoates depolymerases are a group of enzymes responsible for polyhydroxyalkanoates degradation widely found in many microorganisms (Solaiman et al., 2003). They can be divided into extracellular and intracellular depolymerases according to their cellular locations and catalytic mechanisms (Jendrossek & Handrick, 2002). More than 20 genes encoding extracellular polyhydroxyalkanoates depolymerases were cloned from different microbial strains including *Pseudomonas lemoignei* (Schober et al., 2000) and *Ralstonia eutropha* H16 (York et al., 2003), most of which have substrate specificity for PHB. Compared with extracellular polyhydroxyalkanoates depolymerase, the mechanism and regulation of intracellular polyhydroxyalkanoates depolymerase is not well understood. Polyhydroxyalkanoates depolymerase (*phaZ*) of *Pseudomonas putida* KT2442 was an intracellular depolymerase first purified and biochemically characterized by de Eugenio et al. (2007). *In vivo* polyhydroxyalkanoates degradation by intracellular polyhydroxyalkanoates depolymerase was performed by Lee et al. (1999) and Lee & Lee (2003) in an acidic environment; Ren et al. (2005) and Wang et al. (2007) had reported mcl-3HA production in an alkaline environment; and Ruth et al. (2007) had developed 3HA monomer separation and purification technology. It was proven that chiral mcl-3HA monomers can be produced microbially.

Both fatty acid biosynthesis and the β-oxidation pathway can provide precursors to polyhydroxyalkanoates. In *P. putida*, fatty acid degradation is important to provide a precursor for polyhydroxyalkanoates synthesis from octanoic acid, decanoic acid, lauric acid and oleic acid etc. FadL of *E. coli* is an outer membrane protein involved in the binding and transportation of long-chain fatty acids (van den Berg et al., 2004). It is a monomer protein with a long barrel composed of 14 anti-parallel β strands with activity for medium-chain-length fatty acids of C\(_7\)–C\(_{10}\) (Maloy et al., 1981). The fatty acids are activated from a free fatty acid to an acyl-CoA thioester by acyl-CoA synthetase (*fadD*). Acyl-CoA synthetase of *E. coli* has broad substrate specificity from C\(_7\) to C\(_{18}\) (Chen et al., 2005). FadL and FadD jointly promote transport and activation of these hydrophobic compounds to the β-oxidation pathway (DiRusso & Black, 1999).

In this study, for the first time, mcl-3HA monomers were effectively produced by *P. putida* KT2442 jointly expressing *phaZ*\(_{Pst}\), *fadP*\(_{Ec}\) and *fadL*\(_{Pp}\).

**Materials and methods**

**Bacterial strains, media and cultivation conditions**

All the strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were cultivated at 37 °C in Luria–Bertani (LB) medium in which ampicillin (100 μg mL\(^{-1}\)) or kanamycin (50 μg mL\(^{-1}\)) was added if necessary. *Pseudomonas* spp. and their recombinants were grown at 30 °C in LB as an inoculative medium.

For shake flask studies, 5 mL seed culture was inoculated into 95 mL mineral salt (MS) medium containing (L\(^{-1}\)) 9.65 g Na\(_2\)HPO\(_4\), 1.2H\(_2\)O, 1.5 g KH\(_2\)PO\(_4\), 1.0 g (NH\(_4\))\(_2\)SO\(_4\), 0.4 g MgSO\(_4\)·7H\(_2\)O, 1% (v/v) trace element solution supplemented with 20 g L\(^{-1}\) glucose, 20 g L\(^{-1}\) gluconate or 10 g L\(^{-1}\) sodium octanoate as the sole carbon source in a 500-mL baffled shake flask at 30 °C and 200 r.p.m. for 48 h. The composition of trace element solution was the same as reported previously (Qiu et al., 2004).

For fermentation studies, a 6-L fermentor (NBS Bioflo 3000, NJ) with 3 L medium was used containing (L\(^{-1}\)) 2.5 g yeast, 5 g tryptone, 5 g NaCl, 5 g sodium octanoate, 9.65 g Na\(_2\)HPO\(_4\)·12H\(_2\)O, 1.5 g KH\(_2\)PO\(_4\), 1.0 g (NH\(_4\))\(_2\)SO\(_4\), 0.4 g MgSO\(_4\)·7H\(_2\)O and 1% (v/v) trace element solution. Four gram per liter of sodium octanoate and 0.7 g L\(^{-1}\) (NH\(_4\))\(_2\)SO\(_4\) were fed when needed. Temperature and pH were controlled at 30 °C and 7.0, respectively. The agitation speed was gradually increased from 200 to 800 r.p.m. to maintain the dissolved oxygen concentration of 10% air saturation. The aeration rate was 2 L min\(^{-1}\).

**DNA manipulation**

All DNA manipulations in this study were carried out following standard procedures (Sambrook & Russell, 2001). The chromosomal DNA of *E. coli* MG1655, *Pseudomonas aeruginosa* PA01, *P. putida* KT2442 and *Pseudomonas stutzeri* 1317 were isolated by 3S spin genomic DNA (Shenyang Biocolor, Shanghai, China). Plasmid DNA was isolated by the spin mini prep kit (New Yangtze River Co. Ltd, Beijing, China). Plasmid pLZZH13 (Zheng et al., 2006) was a derivative of pBBR1MCS-2 (Kovach et al., 1995) containing the promoter of *R. eutropha* H16 phbCAB synthesis operon with a NdeI site to keep the gene expressed precisely under the promoter. All recombinant plasmids were transformed into *E. coli* S17-1 by electroporation and then introduced into *P. putida* KT2442 by conjugation. The restriction endonucleases, T4 DNA ligase and *ex* *taq* polymerase were
purchased from TaKaRa Co. Ltd (Japan). All the enzymes were used as recommended by the manufacturers.

Construction of plasmids pBBR1MCS-2-cut, pYZPa01, pYZPp01, pYZPs01, pYLPp01, pYDc01, pYZPs03, pYZPs05 and pYZPs06

Plasmid pBBR1MCS-2-cut was a truncated derivative of pBBR1MCS-2 by BstBI digestion and a 946-bp fragment was deleted; then, it self-cyclized to form a new 4098-bp plasmid. The intact plasmid was deleted; then, it self-cyclized to form a new 4098-bp plasmid. The intact plasmid was inserted into pLZZH13 to construct the plasmid by PCR, using its genome as a template; the PCR product was inserted into pLZZH13 to construct the plasmid pYLPp01 to construct plasmid pYDEc01. After that, the fadL gene was inserted into plasmid pYZPs05 to construct plasmid pYZPs06 (Table 1). All of the restriction enzyme sites were underlined and annotated in parentheses. Primers synthesis and gene sequencing were carried out by Invitrogen Co. Ltd (Shanghai, China).

Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strains/plasmids</th>
<th>Relevant characteristics</th>
<th>Sources or references</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli S17-1</td>
<td>recA, harboring the tra genes of plasmid RP4 in the chromosome, proA, thi-1</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td>E. coli MG1655</td>
<td>F- λ− rpl-1 wide type</td>
<td>Sauer et al. (2004)</td>
</tr>
<tr>
<td>P. putida KT2442</td>
<td>hsdR1 hsdM1, Rfr</td>
<td>Bagdasarian et al. (1981)</td>
</tr>
<tr>
<td>P. stutzeri 1317</td>
<td>Wide type, isolated from an oil-contaminated soil, medium-chain-length polyhydroxyalkanoates-producing strain</td>
<td>Chen et al. (2004)</td>
</tr>
<tr>
<td>R. aeruginosa PAO1</td>
<td>Opportunistic pathogen of humans, motile</td>
<td>Shan et al. (2004)</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBBR1MCS-2</td>
<td>Kan^B broad host vector; lacPOZ'</td>
<td>Kovach et al. (1995)</td>
</tr>
<tr>
<td>pBBR1MCS-2-cut</td>
<td>Kan^B pBBR1MCS2 truncated derivative deleted with the lacZ fragment by BstBI digestion and self-cyclized</td>
<td>This study</td>
</tr>
<tr>
<td>pLZZH13</td>
<td>Kan^B pBBR1MCS2 derivative containing the promoter of R. eutropha H16 phbCAB synthesis operon</td>
<td>Zheng et al. (2006)</td>
</tr>
<tr>
<td>pYZPa01</td>
<td>Kan^B pLZZH13 derivative containing phaZ gene from P. aeruginosa PAO1</td>
<td>This study</td>
</tr>
<tr>
<td>pYZPp01</td>
<td>Kan^B pLZZH13 derivative containing P. putida KT2442 phaZ gene</td>
<td>This study</td>
</tr>
<tr>
<td>pYZPs01</td>
<td>Kan^B pLZZH13 derivative containing P. stutzeri 1317 phaZ gene</td>
<td>This study</td>
</tr>
<tr>
<td>pYLPp01</td>
<td>Kan^B pBBR1MCS-2 derivative containing fadL gene from P. putida KT2442</td>
<td>This study</td>
</tr>
<tr>
<td>pYDc01</td>
<td>Kan^B pBBR1MCS-2 derivative containing fadD gene from E. coli MG1655 and fadL gene from P. putida KT2442</td>
<td>This study</td>
</tr>
<tr>
<td>pYDc01</td>
<td>Kan^B pBBR1MCS-2 derivative containing fadD gene from E. coli MG1655 and fadL gene from P. putida KT2442</td>
<td>This study</td>
</tr>
<tr>
<td>pYDc01</td>
<td>Kan^B pBBR1MCS-2 derivative containing fadD gene from E. coli MG1655 and fadL gene from P. putida KT2442</td>
<td>This study</td>
</tr>
</tbody>
</table>
Polyhydroxyalkanoates, 3HHx and 3HO monomer analysis by gas chromatography (GC)

The cells were harvested by centrifugation, washed with ethanol or distilled water and then lyophilized. Polyhydroxyalkanoates contents and cell dry weight (CDW) were measured as described (Zheng et al., 2004).

For 3-hydroxyhexanoate (3HHx) and 3-hydroxyoctanoic acid (3HO) monomer analysis, all fermentation broths were centrifuged at 10 000 g for 10 min. Supernatant (2 mL) was stored at −80 °C in a refrigerator for 1 h and then lyophilized for 36 h, followed by methanolysis. The organic phase was used for GC analysis with decanoic acid as the internal standard (Zheng et al., 2004).

Results

Effects of overexpression of different phaZ genes on cell growth, polyhydroxyalkanoates and 3HA monomer production using P. putida KT2442 as a host

Overexpression of different polyhydroxyalkanoates depolymerases in P. putida KT2442 showed significant effects on cell growth, polyhydroxyalkanoates and 3HA production. After 48 h of cultivation in sodium octanoate MS medium, wild-type P. putida KT2442 accumulated 3.24 g L⁻¹ CDW and 66 wt% polyhydroxyalkanoates while no 3HA monomer was detected (Table 2); P. putida KT2442 (pBBR1MCS-2) accumulated only about 30 wt% polyhydroxyalkanoates, the CDW decreased to 1.93 g L⁻¹ and c. 0.6 g L⁻¹ extracellular 3HA monomers were detected in the supernatants. In strains KT2442 (pYZPa01) and KT2442 (pYZPp01), c. 0.8 g L⁻¹ 3HA accumulated while almost no polyhydroxyalkanoates formed (<1 wt%). Extracellular 3HA (1.37 g L⁻¹) was produced by P. putida KT2442 (pYZPst01) harboring the phaZ gene of P. stutzeri 1317, which includes 21 mol% 3HHx and 79 mol% 3HO (Table 2); P. stutzeri 1317 phaZ gene had shown better ability for 3HA production, and thus it was chosen for further studies.

Interestingly, the overexpression of different phaZ genes seemed not to affect the extracellular 3HA compositions. Neither 3HD nor 3-hydroxydodecanoic acid (3HDD) monomer was detected. This agreed with the fact that the main compositions of polyhydroxyalkanoates accumulated in P. putida KT2442 are 3HO and 3HHx when grown on sodium octanoate as a sole carbon source.

The CDW of phaZ-containing recombinants are lower than that of wild-type P. putida KT2442 (Table 2); however, their residual biomasses were similar when the weight of polyhydroxyalkanoates was excluded (data not shown).

To exclude the effect of antibiotics on 3HA production, P. putida KT2442 (pBBR1MCS-2) cultured without kanamycin was assayed. 3HA could still be detected in its supernatants (data not shown). Plasmid pBBR1MCS-2 have four functional fragments: mob for transfer, kanamycin resistance gene, rep for replication and lacZ. Pseudomonas putida KT2442 (pBBR1MCS-2-cut) without a lacZ fragment was also observed with 3HA accumulation (Table 2). The intracellular polyhydroxyalkanoates contents and extracellular 3HA concentration of P. putida KT2442 (pYZZh13) were similar to those of recombinant P. putida KT2442 (pBBR1MCS-2).

Table 2. Polyhydroxyalkanoates and extracellular 3HA production by Pseudomonas putida KT2442 and its recombinants

<table>
<thead>
<tr>
<th>Strains</th>
<th>CDW (g L⁻¹)</th>
<th>Intracellular polyhydroxyalkanoates (wt%)</th>
<th>Extracellular 3HA ⁺</th>
<th>3HHx (mol%)</th>
<th>3HO (mol%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. putida KT2442</td>
<td>3.24 ± 0.10</td>
<td>66.30 ± 2.72</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>KT2442 (MCS-2)</td>
<td>1.93 ± 0.01</td>
<td>29.95 ± 2.82</td>
<td>0.58 ± 0.15</td>
<td>19.07 ± 0.02</td>
<td>80.93 ± 0.08</td>
</tr>
<tr>
<td>KT2442 (MCS-2-cut)⁷</td>
<td>2.44 ± 0.38</td>
<td>41.43 ± 2.89</td>
<td>0.64 ± 0.26</td>
<td>19.74 ± 0.04</td>
<td>80.26 ± 0.10</td>
</tr>
<tr>
<td>KT2442 (pYZZh13)</td>
<td>2.12 ± 0.15</td>
<td>31.22 ± 4.12</td>
<td>0.61 ± 0.22</td>
<td>20.34 ± 0.01</td>
<td>79.66 ± 0.06</td>
</tr>
<tr>
<td>KT2442 (pYZPa01)</td>
<td>1.15 ± 0.04</td>
<td>0.61 ± 0.03</td>
<td>0.78 ± 0.06</td>
<td>23.04 ± 0.69</td>
<td>76.96 ± 0.69</td>
</tr>
<tr>
<td>KT2442 (pYZPp01)</td>
<td>1.32 ± 0.06</td>
<td>0.45 ± 0.05</td>
<td>0.76 ± 0.08</td>
<td>25.57 ± 1.05</td>
<td>74.43 ± 1.05</td>
</tr>
<tr>
<td>KT2442 (pYZPst01)</td>
<td>1.48 ± 0.06</td>
<td>25.27 ± 3.11</td>
<td>1.37 ± 0.02</td>
<td>21.21 ± 0.75</td>
<td>78.79 ± 0.75</td>
</tr>
<tr>
<td>KT2442 (pYLPp01)</td>
<td>1.91 ± 0.08</td>
<td>33.98 ± 2.08</td>
<td>1.01 ± 0.19</td>
<td>11.89 ± 0.02</td>
<td>88.11 ± 0.17</td>
</tr>
<tr>
<td>KT2442 (pYDEC01)</td>
<td>3.15 ± 0.13</td>
<td>51.50 ± 3.84</td>
<td>0.51 ± 0.15</td>
<td>10.80 ± 0.02</td>
<td>89.20 ± 0.11</td>
</tr>
<tr>
<td>KT2442 (pYDL01)</td>
<td>2.84 ± 0.22</td>
<td>45.88 ± 1.11</td>
<td>1.09 ± 0.08</td>
<td>17.42 ± 0.11</td>
<td>82.58 ± 0.43</td>
</tr>
<tr>
<td>KT2442 (pYZPst03)</td>
<td>1.24 ± 0.08</td>
<td>7.14 ± 1.66</td>
<td>1.79 ± 0.12</td>
<td>20.23 ± 0.63</td>
<td>79.77 ± 0.21</td>
</tr>
<tr>
<td>KT2442 (pYZPst05)</td>
<td>1.19 ± 0.15</td>
<td>16.16 ± 4.68</td>
<td>1.88 ± 0.09</td>
<td>19.52 ± 0.80</td>
<td>80.48 ± 1.25</td>
</tr>
<tr>
<td>KT2442 (pYZPst06)</td>
<td>1.28 ± 0.15</td>
<td>13.55 ± 4.17</td>
<td>2.32 ± 0.21</td>
<td>21.15 ± 0.21</td>
<td>78.85 ± 2.26</td>
</tr>
</tbody>
</table>

*All strains were cultured in MS medium with 10 g L⁻¹ sodium-octanoate and 1 g L⁻¹ (NH₄)₂SO₄ under 30 °C for 48 h. Data were expressed as average value and SD of three parallel experiments.

¹Extracellular 3HA stands for the total 3-hydroxyalkanoic acid concentration including 3HHx and 3HO monomers.

⁷MCS-2-cut is a plasmid that deleted the lacZ fragment of pBBR1MCS-2 by BstBI digestion.

CDW, cell dry weight; 3HHx, extracellular 3-hydroxyhexanoic acid; 3HO, extracellular 3-hydroxyoctanoic acid; MCS-2, plasmid pBBR1MCS-2.
Effects of overexpression of fadL and fadD on cell growth, polyhydroxyalkanoates and 3HA monomer production using *P. putida* KT2442 as a host

Cell growth and polyhydroxyalkanoates accumulation in *P. putida* KT2442 (pYLPp01) were similar to that of strain KT2442 (MCS-2) (Table 2), indicating that fadL overexpression had no obvious effect on polyhydroxyalkanoates production. However, 1.01 g L\(^{-1}\) 3HA was detected in the broth, suggesting that more fatty acid metabolic flux promoted 3HA production.

The polyhydroxyalkanoates contents and CDW of *P. putida* KT2442 (pYDDe01) reached 51.50 wt% and 3.15 g L\(^{-1}\), respectively, with 0.51 g L\(^{-1}\) 3HA monomers found in the broth (Table 2). Little residual sodium octanoate was left after 48 h of cultivation (data not shown), which suggested that overexpression of the fadD gene compensated for the negative effect of pBBR1MCS-2, allowing the strains to recover to a normal growth. *Pseudomonas putida* KT2442 (pYDL01) containing both fadL\(_Pp\) and fadD\(_E\) genes produced 2.84 g L\(^{-1}\) CDW and 46 wt% polyhydroxyalkanoates, accompanied by over 1 g L\(^{-1}\) 3HA accumulation.

Effects of coexpression of fadL, fadD andphaZ genes on cell growth, polyhydroxyalkanoates and 3HA monomer production using *P. putida* KT2442 as a host

*Pseudomonas putida* KT2442 (pYZPst03) and *P. putida* KT2442 (pYZPst05) accumulated 1.8 and 1.9 g L\(^{-1}\) extracellular 3HA, respectively (Table 2). Remarkably, joint overexpression of fadD, fadL and phaZ in *P. putida* KT2442 (pYZPst06) achieved the highest extracellular 3HA production of 2.3 g L\(^{-1}\) (Table 2). The composition of 3HA monomers produced by all recombinants was always around 80 mol% 3HO and 20 mol% 3HHx, similar to the phaZ overexpression solely. The intracellular polyhydroxyalkanoates content of *P. putida* KT2442 (pYZPst06) was only 13 wt%, indicating that the extracellular 3HA is dominant over intracellular granules of polyhydroxyalkanoates (Table 2).

*Pseudomonas putida* KT2442 could accumulate polyhydroxyalkanoates from glucose (Huijberts et al., 1992), therefore an attempt was made to produce 3HA using some unrelated carbon sources including glucose, sodium and gluconate. Using glucose or gluconate as a carbon source, *P. putida* KT2442 (pYZPst06) accumulated < 1% polyhydroxyalkanoates, and no extracellular 3HA was detected in the supernatant (Table 3). Thus, sodium octanoate was chosen for fermentation studies.

Effects of ammonium sulfate concentration on 3HA monomer production using *P. putida* KT2442 (pYZPst06) as a host

The production of 3HA was different in *P. putida* KT2442 (pYZPst06) when it grew on MS medium supplemented with (NH\(_4\)\(_2\))\(_2\)SO\(_4\) concentrations of 0.5, 1.0 and 2.0 g L\(^{-1}\), respectively. With an increase of the (NH\(_4\)\(_2\))\(_2\)SO\(_4\) concentration, CDW production increased, and yet intracellular polyhydroxyalkanoates levels were observed to decrease. However, the medium containing 1.0 g L\(^{-1}\) (NH\(_4\)\(_2\))\(_2\)SO\(_4\) produced the highest extracellular 3HA (Table 4). No significant change was observed among compositions of 3HA with different (NH\(_4\)\(_2\))\(_2\)SO\(_4\) concentrations in the media; their compositions were maintained around 20 mol% 3HHx and 80 mol% 3HO. The residual sodium octanoate was too low to be detected when the (NH\(_4\)\(_2\))\(_2\)SO\(_4\) concentration was 2 g L\(^{-1}\), while about 6–7 g L\(^{-1}\) residual sodium octanoate was left in the medium when the (NH\(_4\)\(_2\))\(_2\)SO\(_4\) concentration was 1 or 0.5 g L\(^{-1}\) (Table 4).

**3HA monomer production by *P. putida* KT2442 (pYZPst06) grown in a 6-L fermentor**

The production of 3HA by *P. putida* KT2442 (pYZPst06) was examined in a 6-L fermentor containing 3 L medium. Kim (2002) reported that over 4 g L\(^{-1}\) sodium octanoate...
on 21 January 2018

Table 4. Shake flask study of extracellular 3HA production by Pseudomonas putida KT2442 (pYZPst06)* in the presence of different ammonium sulfate concentrations

<table>
<thead>
<tr>
<th>(NH₄)₂SO₄ Conc. (g L⁻¹)</th>
<th>CDW (g L⁻¹)</th>
<th>Intracellular polyhydroxyalkanoates (wt%)</th>
<th>Extracellular 3HA†</th>
<th>Residual Na-octanoate‡ (g L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>(g L⁻¹)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>3HHx (mol%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3HO (mol%)</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>0.88 ± 0.06</td>
<td>15.40 ± 0.98</td>
<td>0.76 ± 0.08</td>
<td>7.02 ± 0.10</td>
</tr>
<tr>
<td>1.0</td>
<td>1.28 ± 0.15</td>
<td>13.55 ± 4.17</td>
<td>2.32 ± 0.21</td>
<td>6.77 ± 2.64</td>
</tr>
<tr>
<td>2.0</td>
<td>2.75 ± 0.12</td>
<td>9.38 ± 4.93</td>
<td>1.28 ± 0.08</td>
<td>80.04 ± 0.09</td>
</tr>
</tbody>
</table>

*Plasmid pYZPst06 contains fatty acid degradation genes fadD and fadL as well as polyhydroxyalkanoates depolymerase gene phaZ.
†Extracellular 3HA stands for the total 3-hydroxyalkanoic acid concentration including extracellular 3HHx and 3HO monomers.
‡Residual Na-octanoate was detected by GC using its methyl esters.

Inhibited the growth of P. oleovorans. Thus, 4 g L⁻¹ sodium octanoate and 0.7 g L⁻¹ (NH₄)₂SO₄ were supplemented as a feed medium when needed during the fermentation process.

CDW of P. putida KT2442 (pYZPst06) grew rapidly to 6.34 g L⁻¹ after 12 h of fermentation; the cells reached a stationary phase in the subsequent 16 h. The polyhydroxyalkanoates content increased slowly from 15 wt% CDW at 12 h to nearly 35 wt%/CDW at 20 h, and remained stable later. The residual sodium octanoate decreased from 5 to < 2 g L⁻¹ after 8 h of fermentation. During the octanoate feeding process, the concentration of residual sodium octanoate fluctuated between 2 and 4 g L⁻¹.

Finally, 8 g L⁻¹ CDW containing 32 wt% polyhydroxyalkanoates was obtained. Extracellular 3HA monomer levels reached 5.8 g L⁻¹ (Fig. 1), which was more than twice the shake flask result (Table 2). The composition of 3HA monomers consisted of around 80 mol% 3HO and 20 mol% 3HHx in the fermentation supernatants (data not shown).

### Discussion

To produce extracellular mcl-3HA, a metabolic pathway for 3HHx and 3HO production was reinforced via simultaneous expression of the putative long-chain fatty acid transport protein (fadL) of P. putida KT2442, acyl-CoA synthetase (fadD) gene of E. coli MG1655 and polyhydroxyalkanoates depolymerase (phaZ) of P. stutzeri 1317 in the P. putida KT2442 host. The pathway begins with the cross-membrane transport of free fatty acids by FadL, followed by β-oxidation from fatty acids to fatty acid CoA thioesters in the cytoplasm by FadD. The reinforced β-oxidation of fatty acids can supply more precursors for polyhydroxyalkanoates synthesis. Intracellular polyhydroxyalkanoates will be degraded by polyhydroxyalkanoates depolymerase encoded by phaZ and 3HA released. In this study, phaZ cloned from P. stutzeri 1317 was found to be more effective than other polyhydroxyalkanoates depolymerases in depolymerizing the polyhydroxyalkanoates to 3HA (Table 2). Further study indicated that phaZ overexpression reduced the ability of P. putida KT2442 to produce polyhydroxyalkanoates (data not shown), as indicated in P. putida U reported by Sandoval et al. (2005). An enhanced 3HA production was observed when fadD or fadL was coexpressed with phaZ in P. putida KT2442. The highest 3HA production of 2.3 g L⁻¹ was obtained in the shake flask of P. putida KT2442 (pYZPst06) grown on the MS medium with sodium octanoate as the sole carbon source for 48 h; the coexpression of fadD, fadL and phaZ was the obvious reason for the efficient 3HA production (Table 2).

In addition, the selection of carbon sources and ratio of carbon to nitrogen (C/N) can affect 3HA production efficiency (Tables 3 and 4). In many cases, nitrogen limitation or a high C/N ratio promotes polyhydroxyalkanoates synthesis (Durner et al., 2000), which was confirmed in this study.
However, no linear relationship was found between C/N ratio and 3HA production, although an appropriate C/N ratio is very important for effective production of 3HA. The scale-up of 3HA production in a 6-L fermentor yielded a 5.8 g L\(^{-1}\) 3HA monomer. Although the initial products of PhaZ could be dimers, monomers or their mixture, the final PhaZ products were all monomers after further incubation and hydrolysis (Radice et al., 2007). This study demonstrated for the first time that fadD and fadL play an important role in \(\beta\)-oxidation of fatty acids in \(*P. putida*\), and they are very useful for extracellular 3HA production.

Most of the phaZ genes of *Pseudomonas* species were located between polyhydroxyalkanoates synthases phaC1 and phaC2. Little has been known about their phaZ expression under normal growth conditions. No extracellular 3HA was produced by wild-type *P. putida* KT2442, possibly due to the strict regulation of depolymerase activity under normal growth conditions. *Pseudomonas putida* KT2442 harboring the series plasmids based on pBBR1MCS-2 showed a negative effect on cell growth and polyhydroxyalkanoates accumulation, but was positive for extracellular 3HA production (Table 2), although mechanisms for 3HA leakage into the extracellular space required further investigation.

In fermentor studies, the extracellular 3HA amount was found to be fluctuated, indicating that 3HA could be consumed by the cells again if the cells grew well, which is a major problem when dealing with the production of 3HA monomers through *in vivo* depolymerization (Lee et al., 1999). Production of 3HA may be more effective if 3HA is removed continuously during the fermentation process.

A previous study showed that fadBA will be induced in *E. coli* when fadD is overexpressed (Zhang et al., 2006). Excessive amounts of medium-chain acyl-CoA would probably bind with the transcriptional repressor FadR to remove the inhibition of fatty acid \(\beta\)-oxidation (Zhang et al., 2006). This assumption was helpful for illustrating the effect on 3HA production under fadD overexpression in *P. putida* KT2442. Substrates of FadD are mainly medium-chain-length fatty acids including C8, C10 and C12 (Maloy et al., 1981). FadL of *P. putida* KT2442 is a putative long-chain fatty acid transporter. A previous study showed that octanoate uptake by *P. oleovorans* was a translocation process rather than surface adsorption (Toscano & Hartline, 1973). In our study, we partially proved its fatty acid transportation although we have not proven its high affinity for long-chain fatty acids. Overexpression on fadD and fadL enhances the endurance of a strain to high a concentration of fatty acids, improves the fatty acid utilization and leads to more flux of \(\beta\)-oxidation to polyhydroxyalkanoates synthesis; therefore, more substrates would be provided to PhaZ, and then more 3HA would be produced (Table 2 and Fig. 1).

Wang et al. (2007), Ruth et al. (2007) and Ren et al. (2005) reported that *P. putida* could secrete the 3HA monomer under an alkaline condition (pH 10–11). They speculated that phaZ was the critical gene responsible for the hydrolysis of polyhydroxyalkanoates during this process; however, only a few references (Lee & Lee, 2003; Sandoval et al., 2005) were found on polyhydroxyalkanoates monomer production via phaZ overexpression directly.

Not all the monomers produced by *in vivo* depolymerization were pure R-enantiomers. Once depolymerized, some R-enantiomers may be isomerized to form their S-enantiomers; as a result, a racemic mixture may be released into the broth (Sandoval et al., 2005). However, the 3HA produced via polyhydroxyalkanoates resuspension in different buffers were pure R-enantiomers (Lee et al., 1999; Ren et al., 2005; Ruth et al., 2007). Owing to limited conditions, the configuration of the 3HA in our study was not determined.

In summary, a new method using phaZ, together with fadD and fadL, was developed to produce extracellular 3HA effectively under neutral pH conditions that prevents the cells from undergoing lysis (Wang et al., 2007). As the PhaZ substrate specificity on medium-chain-length fatty acids (Radice et al., 2007), this method may also be useful for the production of other mcl-3HA containing aromatic or unsaturated groups.

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