RESEARCH PAPER

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Biosynthesis of poly(3-hydroxypropionate) from glycerol using engineered *Klebsiella pneumoniae* strain without vitamin B_{12}

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Keywords: glycerol, *Klebsiella pneumoniae*, poly(3-hydroxypropionate), vitamin B_{12}, 3-hydroxypropionate

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Poly(3-hydroxypropionate) (P3HP) is a biodegradable and biocompatible thermoplastic. Previous studies demonstrated that engineered *Escherichia coli* strains can produce P3HP with supplementation of expensive vitamin B_{12}. The present study examined the production of P3HP from glycerol in the recombinant *Klebsiella pneumoniae* strain, which naturally synthesizes vitamin B_{12}. The genes glycerol dehydratase and its reactivation factor (*dhaB123*, *gdrA,* and *gdrB* from *K. pneumoniae*), aldehyde dehydrogenase (*aldH* from *E. coli*) were cloned and expressed in *K. pneumoniae* to produce 3-hydroxypropionate (3HP), with 2 genes (*dhaT* and *yqhD*) for biosynthesis of 1,3-propanediol were deleted. To obtain P3HP production, propionyl-CoA synthetase (*prpE* from *E. coli*) and polyhydroxyalkanoate synthase (*phaC* from *Ralstonia eutropha*) were introduced. Under the appropriate aeration condition, the cell yield and P3HP content were 0.24 g/L and 12.7% (wt/wt [cell dry weight]) respectively along with 2.03 g/L 3HP after 48 h cultivation. Although the yield is relatively low, this study shows the feasibility of producing P3HP in *K. pneumoniae* from glycerol without vitamin B_{12} for the first time. The results also suggest that the aeration conditions should be optimized carefully for the efficient production of P3HP.

Introduction

As the main byproduct of biodiesel, about 10 tons of crude glycerol is produced with 100 tons of biodiesel produced via transesterification of vegetable oils or animal fats.[1] With the rapid development of the biodiesel industry, glycerol has become an inexpensive and abundant carbon source. Glycerol is both a cell metabolite and a carbon substrate for growth.[2] It can be converted into many valuable products, such as 1,3-propanediol,[3] lactate,[4] succinate,[5] 3HP,[6] and polyhydroxyalkanoates (PHAs).[1]

Poly(3-hydroxypropionate) (P3HP) is one kind of PHA exhibiting high rigidity, ductility, and exceptional tensile strength in drawn films, and was regarded as one of the alternatives to petrochemical-derived plastic.[1] Thus far, P3HP cannot be produced by any native bacterial strains. When some structurally-related carbon sources like 3HP, α,ω-alkanediols, acrylate, γ-butyrolactone or 1,3-propanediol were added, PHAs containing the 3HP monomers could be produced by some microbes like *Alcaligenes eutrophus*,[7] *Alcaligenes latus*,[8] *Ralstonia eutropha*,[9] *Methylobacterium*,[10] and engineered *Escherichia coli.*[11] However, the addition of these expensive precursors increased the production cost and restricted the further development.

The first attempt to produce PHAs containing the 3HP monomer from unrelated carbon sources was reported in 2009 by engineered *Cupriavidus necator*, and the product was P(3HP-co-3HB).[12] In recent years, P3HP was produced successfully from glycerol using engineered *E. coli* strains. However, *E. coli* requires the addition of vitamin B_{12} as the coenzyme of glycerol dehydratase employed in the pathway for conversion glycerol into 3-hydroxypropioaldehyde.[13] Vitamin B_{12} is expensive and economically not feasible.

Klebsiella pneumoniae is one kind of microorganism studied sufficiently which can produce vitamin B_{12} naturally.[14] It can grow well on glycerol as the sole carbon and energy source. In this study, recombinant strains of *K. pneumoniae* were developed and evaluated, which can produce P3HP from glycerol (**Fig. 1**). To improve the yield of 3HP, Q1546 was developed by disrupting 1,3-PDO oxidoreductases *dhaT* and *yqhD*, and overexpression of aldehyde dehydrogenase *aldH* from *E. coli*, glycerol dehydratase *dhaB123* and glycerol dehydratase reactivation factor *gdrAB* from *K. pneumoniae*. In order to obtain P3HP, plasmid pBAD18-pp with propionyl-CoA synthetase *prpE* from *E. coli* and PHA synthase *phaC* from *R. eutropha* H16 was transformed into the above 3HP producing *K. pneumoniae*. The recombinant strain Q1643 was cultured in vitamin B_{12} free medium under a range of aeration conditions. The effects of aeration on 3HP and P3HP production were compared. This study shows the potential and limitation of

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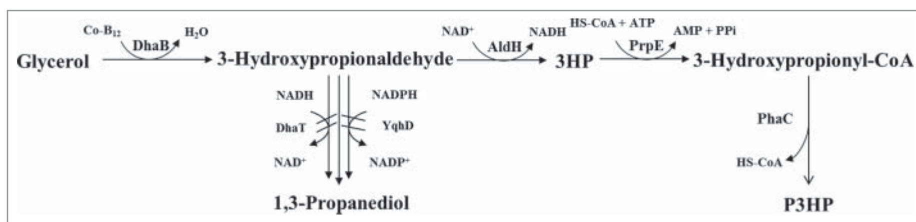

Figure 1. Metabolic pathway and genetic modification strategy for P3HP production by recombinant *K. pneumoniae* strain from glycerol. DhaB: glycerol dehydratase; AldH: aldehyde dehydrogenase; PrpE: propionyl-CoA synthetase; PhaC: polyhydroxyalkanoate synthetase; DhaT and YqhD: 1,3-propanediol oxidoreductases.

K. pneumoniae in the production P3HP from glycerol in vitamin B_{12} free medium.

Results and discussion

Confirmation of target proteins in *Klebsiella pneumoniae*

Glycerol can be converted into 3-hydroxypropionaldehyde (3-HPA) through catalysis by glycerol dehydratase in *K. pneumoniae*. And then 3-HPA is reduced to 1,3-PDO by 1,3-PDO oxidoreductases or oxidized to 3HP by aldehyde dehydrogenase. The wild type *K. pneumoniae* did not produce 3HP at a significant level during glycerol fermentation and 1,3-PDO accumulated simultaneously.[15] To improve 3HP production, 1,3-PDO synthesis was disrupted by deleting *dhaT* and *yqhD*, and genes related to 3HP synthesis were cloned and overexpressed in *K. pneumoniae* ATCC25955.

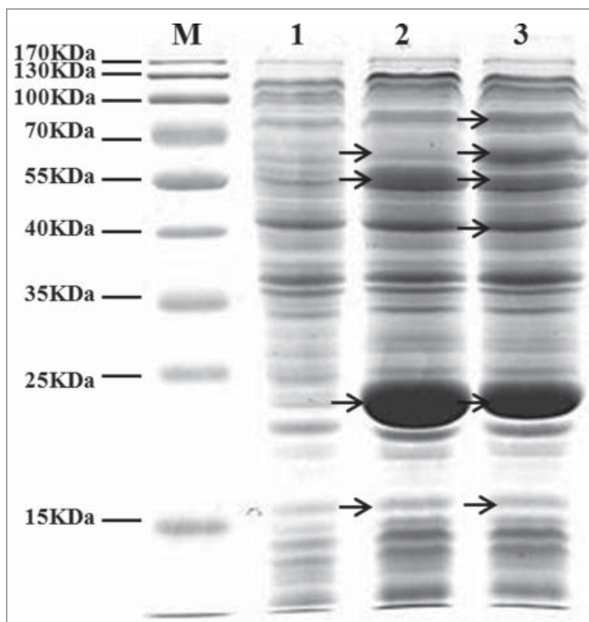

Figure 2. SDS-PAGE analysis of cell-free soluble extract of Q1533, Q1546 and Q1643. The arrows indicate PrpE～70KDa, $DhaB_1$ and GdrA～62KDa, AldH～55KDa, PhaC～40KDa, $DhaB_2$～21KDa, $DhaB_3$ and GdrB～16KDa. M-protein maker, 1-Q1533, 2-Q1546 and 3-Q1643.

The expression of cloned genes in Q1546 and Q1643 after growing in LB medium was analyzed. With L-arabinose induced, all the targeted proteins were observed as a prominent band with the expected molecular on SDS-PAGE (**Fig. 2**). The samples showed the presence of the target protein bands at 62 kDa (DhaB1 and GdrA), 55 kDa (AldH), 21 kDa (DhaB2), and 16 kDa (DhaB3 and GdrB), whereas the Q1643 cultures also showed a prominent band at 70 kDa (PrpE), and 40 kDa (PhaC). The results demonstrated that these proteins were properly expressed in recombinant strains.

3HP production by different recombinant strains

The production of 3HP in different recombinant strains were compared (**Fig. 3**). In this study, only 0.63 ± 0.07 g/L 3HP was produced by the control strain Q1188. To improve the 3HP biosynthesis, genes related to 1,3-PDO synthesis, *dhaT* and *yqhD*, were knocked out using suicide vector pRE112. After 48 h cultivation, the *dhaT* mutant strain Q1189 produced 1.51 ± 0.19 g/L 3HP and *dhaT yqhD* double mutant strain Q1153 accumulated 2.07 ± 0.11 g/L 3HP. Deleting of *dhaT* and *yqhD* enhanced the glycerol carbon flux toward to 3HP. The results were consistent with the previous report.[14]

When target proteins were overexpressed, 3HP production was dramatically improved, Q1545 produced 2.29 ± 0.26 g/L 3HP and Q1546 produced 4.01 ± 0.21 g/L 3HP which were improved to 3.64 and 6.37 times from the production observed using the control strain Q1188 respectively. This shows that overexpression of the genes related to the synthesis of 3HP can play an important role in improving the production. As Q1546 exhibited the highest production, it was used as a host for producing P3HP in further study.

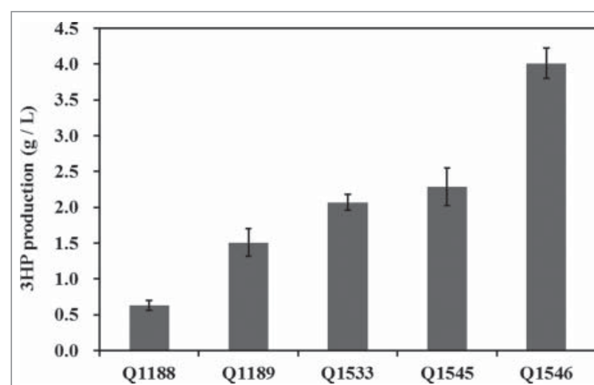

Figure 3. Production of 3HP by different recombinant strains. The strains were grown in minimal medium under shake flask condition in triplicate, and standard deviation was shown.

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P3HP accumulation and analysis

For P3HP production, plasmid pBAD18-pp harboring propionyl-CoA synthetase (*prpE* from *E. coli*, which can convert 3HP to 3-hydroxypropionyl CoA), and polyhydroxyalkanoate synthase (*phaC* from *R. eutropha*, which can polymerize 3-hydroxypropionyl CoA into P3HP) was transferred into Q1546 and Q1643 was obtained. Q1643 was cultivated with glycerol as the sole carbon source. After 48 h cultivation, the cells were harvested and PHA was extracted with hot chloroform from lyophilized cells. The extracted PHA was dissolved in deuterated chloroform (50 mg/ml) to confirm the chemical structure by NMR analysis at room temperature with tetramethylisilane (TMS) as internal chemical shift standard. The characteristic ^{1}H and ^{13}C-NMR spectrum of the sample is presented in **Figure 4**. The integrated peak areas of ^{1}H-NMR spectrum were found to be 1, 1 respectively for the CH2(2), CH2(3). A similar identification has been already performed for poly (3-hydroxypropionate).[13] **Figure 4** shows also the ^{13}C-NMR spectrum of P3HP, the chemical shifts are similar to those obtained by previous study.[13]

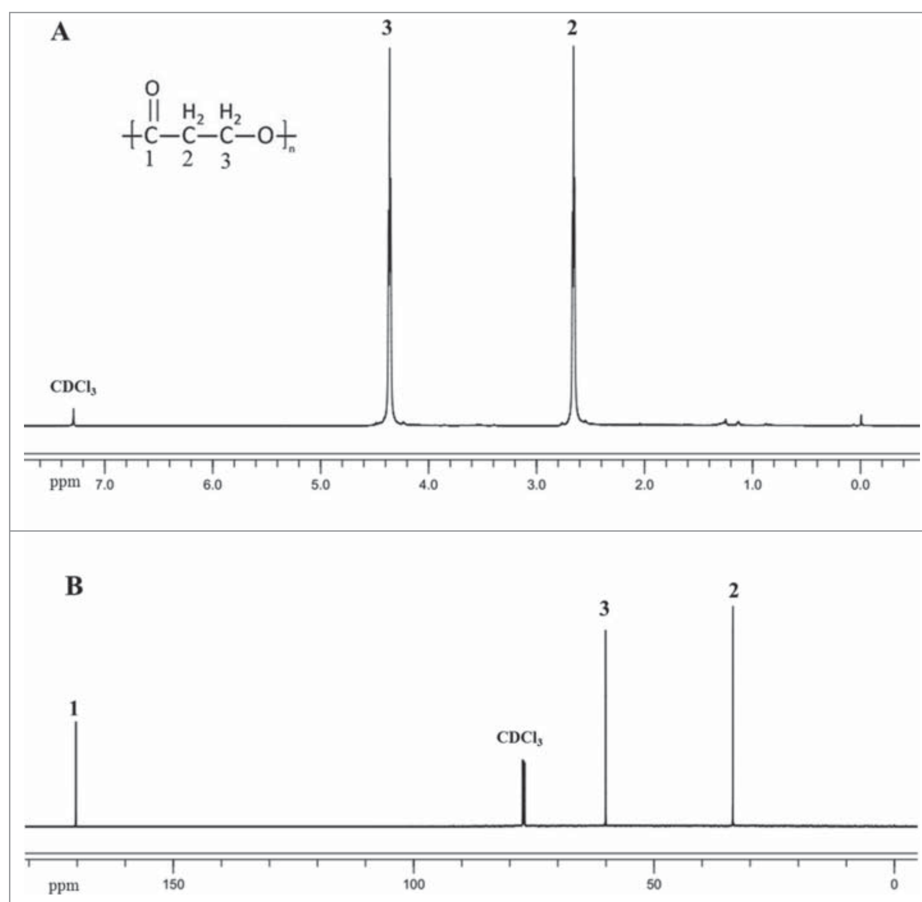

Figure 4. The 600 MHz ^{1}H(A) and ^{13}C(B) NMR spectra of P3HP synthesized by Q1643 in $CDCl_3$ solution. The chemical shift assignment for each proton and carbon resonance was showed.

Effect of aeration on P3HP production

The aeration condition plays an important role in many metabolites production. For this reason, the effect of aeration on P3HP production in the recombinant strain Q1643 was examined by varying the shaking speed (50, 100, or 200 rpm) with a working volume 100 mL in a 250 mL flask culture (**Table 1**). With the speed of 50 rpm, the P3HP production and CDW were at the minimum level. With the increase of aeration, the P3HP production and cell density increased, and reached 0.24 g/L and 1.89 g/L respectively at the shaking speed of 100 rpm. At the same time, 2.03 g/L 3HP was produced under such aeration condition. The enhanced P3HP production could be attributed to improved 3HP production, and the enhanced 3HP production at higher aeration can be attributed to the higher *aldH* expression levels.[16] Compared with the cultures under poorer aeration conditions, the cell density (3.57 g/L) was improved dramatically under fully aerobic condition (200 rpm). In contrast, further enhanced aeration did not result in enhanced production, only trace amounts of P3HP (0.06 g/L) and a little 3HP (0.65) were produced. It was presumably caused by the repression of *dha* operon involving glycerol dehydrogenase, glycerol dehydratase and PDOR.[17,18]

In the engineered metabolic pathway of this study, 3HP is the precursor of P3HP. However, under all the 3 aeration conditions, P3HP production is only about 10 percent of 3HP production. The quite low efficiency of the conversion from 3HP to P3HP might due to the low enzyme activity of PrpE. The similar phenomenon was observed when propionyl-CoA synthase expressed in *Cupriavidus necator*.[12] On the other hand, PrpE is the key enzyme in propionate catabolism which catalyzes propionate into propionyl-CoA. Compared with propionate, 3HP is maybe not a suitable substrate for PrpE. The modification of PrpE and using other propionyl-CoA synthetase from different sources like *Salmonella typhimurium*[19] should be tried in the future for improving the production of P3HP.

Table 1. Production of P3HP under different aeration

Aeration (rpm)	CDW (g/L)	P3HP (g/L)	P3HP content (%)	3HP (g/L)
50	0.46 ± 0.05	0.08 ± 0.01	17.39	1.73 ± 0.12
100	1.89 ± 0.17	0.24 ± 0.04	12.70	2.03 ± 0.08
200	3.57 ± 0.31	0.06 ± 0.03	1.68	0.65 ± 0.09

Table 2. Bacterial strains, plasmids, and primers used in this study

Strain, plasmid and primers	Description	Source
Strains		
E. coli DH5α	F^- supE44 ΔlacU169 (Φ80 lacZ ΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1, cloning host	Invitrogen
E. coli BL21	F^- ompT $hsdS_B$ (r_B^- m_B^-) gal dcm (DE3), source of aldH	Invitrogen
Q1188	K. pneumoniae ATCC25955	ATCC
Q1189	K.pneumoniae ATCC25955 ΔdhaT mutant	Ref. 4
Q1533	K.pneumoniae ATCC25955 ΔdhaTΔyqhD mutant	Ref. 4
Q1545	Q1189pEdg	this study
Q1546	Q1533pEdg	this study
Q1643	Q1546pBAD18-pp	this study
Plasmids		
pBAD33	AraBAD promotor, chloramphenicol resistant	NBRP-E.coli at NIG
pBAD18	AraBAD promotor; kanamycin resistant	NBRP-E.coli at NIG
pBAD33-aldH	Plasmid pBAD33 with aldH, chloramphenicol resistant	this study
pHP304	Plasmid pET21a with phaC and prpE, ampicillin resistant	Ref. 20
pEdg	Plasmid pBAD33 with aldH, dhaB123 and gdrAB, chloramphenicol resistant	this study
pBAD18-pp	Plasmid pBAD18 with phaC and prpE, kanamycin resistant	this study
Primers	Sequence(5′-3′)	Restriction enzymes
aldH cloning		
323	CATGAGCTCGATAGACGTGAAACAGGAGTC	SacI
324	CAGCTCTAGATCAGGCCTCCAGGCTTATCC	XbaI
dhaB123 cloning		
325	CAGCTCTAGAGGATTTCACCTTTTGAGCCGATG	XbaI
358	TTAACGGCATGCTGACCTCCGCTTAG	
gdrAB cloning		
357	GCGGAGGTCAGCATGCCGTTAATAG	
124	CAGAAGCTTCAGTTTCTCTCACTTAACG	HindIII

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Conclusion

K. pneumoniae strain was metabolically engineered and showed the potential of being able to produce P3HP from glycerol without external addition of coenzyme Vitamin B_{12}. The aeration conditions played a vital role in P3HP production and cell growth. Under controlled aeration (100 rpm, 100 mL working volume in 250 mL non-baffled flasks), Q1643 produced 0.24 g/L P3HP which accounted for 12.7% content of cell dry. This study shows the feasibility of producing P3HP from glycerol using *K. pneumonia*e without vitamin B_{12} for the first time. Further investigation and metabolic engineering approaches are required to improve the P3HP yield in *K. pneumoniae* and make it commercially viable.

Materials and Methods

Strains and plasmids construction

All the bacterial strains, plasmids and primers are listed in **Table 2**. *E. coli* DH5α was used for plasmid cloning and maintenance. Standard recombinant DNA procedures were used for gene cloning and plasmid isolation. The gene *aldH* was amplified from *E. coli* BL21, and cloned into *SacI* and *XbaI* restriction sites of the pBAD33 vector to generate pBAD33-aldH. PCR fragment *dhaB123-gdrAB* was obtained by overlapping PCR after *dhaB123* and *gdrAB* were amplified by PCR from the genomic DNA isolated from *K. pneumoniae* ATCC25955. The fragment was digested with *XbaI* and *HindIII* and then ligated between the same restriction sites of pBAD33-aldH to construct pEdg. The pBAD18-pp plasmid was constructed by inserting *phaC-prpE* fragment (enzymatic digestion with *HindIII* and *SalI* from pHP304 constructed by our previous study[20]) into pBAD18. The growth conditions for construction and the methods for developing *K. pneumoniae* mutant strains were carried out as described by our previous study.[4]

Protein expression

To confirm that the recombinant strain had been properly constructed, the strains Q1533, Q1546, and Q1643 were cultured in LB medium at 37 °C and induced at an OD_{600} of 0.6 ∼0.8 by adding 0.05% L-arabinose. The temperature was set at 30 °C after induced. The cells were harvested 3h after induction and centrifuged at 12000 rpm at 4 °C for 10min. The cell pellets were washed twice with 100 mM potassium phosphate buffer (pH7.0) and resuspended in the same buffer. The cells were lysed by Ultrasonic Cell Disruption System and centrifuged at 15000 rpm for 10min and the supernatants were used for SDS-PAGE. Protein expression was examined on SDS-PAGE under denaturing conditions and Coomassie brilliant blue was used to stain the proteins.

Shake flask cultivation

Shake flask cultivation was carried out with 100 mL working volume in 250 mL non-baffled flasks at 37 °C in an orbital incubator shaker at 150 rpm for producing 3HP. To examine the effect of aeration on the P3HP production, the recombinant strain Q1643 was examined by varying the shaking speed (50, 100, 200 rpm).

All the cultures were induced at $0.6\sim0.8OD_{600}$ with 0.05% (w/v) L-arabinose for producing targeted product. After induced, the temperature was set at 30 °C for further cultivation. Ammonia was supplemented for pH control every 12 h along with antibiotics added. 20 g/L glycerol was added into the medium after 24h. The preculture and fermentation medium used in this study contained the following components (per liter of deionized water): glycerol, 20 g; yeast extract, 3 g; citric acid, 0.42 g; KH_2PO_4, 2 g; K_2HPO_4, 1.6 g; NH_4Cl, 5.4 g; $MgSO_4{\cdot}7H_2O$, 0.2 g; and 1 mL of trace elements solution ($FeCl_3{\cdot}6H_2O$, 5 g; $MnCl_2{\cdot}4H_2O$, 2 g; $ZnCl_2$, 0.684 g; $CoCl_2{\cdot}6H2O$, 0.476 g; $CuCl_2{\cdot}2H_2O$, 0.17 g; H_3BO_3, 0.062 g; $Na_2MoO_4{\cdot}2H_2O$, 0.005 g; and 10 mL of concentrated HCl per liter).

Analytical methods

The samples were withdrawn to determine the 3HP and P3HP yield after induced 48 h. All shake experiments were carried out in triplicates. The concentration of 3HP was determined by HPLC using the method reported by Raj et al.[16] The cells were harvested after cultivation by centrifugation and lyophilized. PHA was extracted from the cells with hot chloroform in a Soxhlet apparatus for 10 h, and precipitated by ice-cold ethanol.[21] After ethanol and chloroform volatilized completely at room temperature, the polymer structure was analyzed by NMR using an Advanced III 600 NMR Spectrometer (Bruker, Switzerland). Both ^{13}C NMR and ^{1}H NMR spectra of the sample were measured at 25 °C in deuterated chloroform ($CDCl_3$) (50 mg/mL) with Tetramethylisilane (TMS) as internal chemical shift standard.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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