Construction of Recombinant *E. coli* Expressing Fusion Protein to Produce 1,3-Propanediol

Rosarin Rujananon, Poonsuk Prasertsan, Amornrat Phongdara, Tanate Panrat, Jibin Sun, Sugima Rappert, An-Ping Zeng

**Abstract**—In this study, a synthetic pathway was created by assembling genes from *Clostridium butyricum* and *Escherichia coli* in different combinations. Among the genes were *dhaB1* and *dhaB2* from *C. butyricum* VP1718 coding for glycerol dehydratase (GDH) and its activator (GDHAc), respectively, involved in the conversion of glycerol to 3-hydroxypropionaldehyde (3-HPA). The *yqhD* gene from *E. coli* BL21 was also included which codes for an NADPH-dependent 1,3-propanediol oxidoreductase isoenzyme (PDORI) reducing 3-HPA to 1,3-propanediol (1,3-PD). Molecular modeling analysis indicated that the conformation of fusion protein of YOHD and DHA1 was favorable for direct molecular channeling of the intermediate 3-HPA. According to the simulation results, the *yqhD* and *dhaB1* gene were assembled in the upstream of *dhaB2* to express a fusion protein, yielding the recombinant strain *E. coli*BL21 (DE3)/pET22b:::*yqhD-dhaB1-dhaB2* (strain BP41Y3). Strain BP41Y3 gave 10-fold higher 1,3-PD concentration than *E. coli*BL21 (DE3)/pET22b:::*yqh-dhaB1-dhaB2* (strain BP31Y2) expressing the recombinant enzymes simultaneously but in a non-fusion mode. This is the first report using a gene fusion approach to enhance the biological conversion of glycerol to the value added compound 1,3-PD.

**Keywords**—Recombinant *E. coli*, 1,3-propanediol, glycerol, fusion protein.

I. **INTRODUCTION**

1,3-Propanediol (1,3-PD) is a valuable chemical molecule for many synthetic reactions, particularly as a monomer to produce new polyester called poly(propylene terephthalate) (PET). This has superior properties such as good light stability, biodegradability and improved elasticity for the fiber industry [1], [2]. Over 10¹⁵ ton of 1,3-PD are produced annually [3] and will be in demand more in the future. 1,3-PD is mainly produced through chemical synthesis from petroleum derivatives such as ethylene oxide and acrolein. However, the chemical processes for producing 1,3-PD has low selectivity, conversion takes place under dangerous conditions, high cost and power consumption. In addition, it is not environment-friendly [4]. Therefore, the attention has focused on developing an environmentally friendly approach for producing 1,3-PD. The fermentation of glycerol to 1,3-PD has been studied with several microorganisms including *Klebsiella pneumoniae* [5,6], *Citrobacter freundii* [7], *C. butyricum* [8] which is carried out in two sequential enzymatic steps. Glycerol is transported into the cell through the glycerol facilitator [9]. It is dehydrated by coenzyme B₁₂-dependent glycerol dehydratase (GDH), encoded by *dhaB*, to form 3-hydroxypropion-aldehyde (3-HPA) [10], [7] which is reduced to 1,3-PD by 1,3-propanediol oxidoreductase (PDOR), encoded by *dhaT* in presence of reducing power NADH [11], [12], then excreted into the medium [9]. The key limitation step of this pathway is the dehydration of glycerol to 3-HPA by GDH which normally requires coenzyme B₁₂ as a cofactor. This is a constraint to the application of some of the microorganisms in industry because it is necessary to add an excessive amount of a high-cost molecule, vitamin B₁₂, to the culture medium. In 2000, Laffend and Nagarajan [13] reported that recombinant *E. coli* harboring the *dhaB* and *dhaT* converted glycerol to 1,3-PD with lower titers. This probably resulted from the activity of PDOR not being sufficient to catalyze the conversion of 3-HPA to 1,3-PD completely, leading to an accumulation of 3-HPA. This can cause damage to the DNA, resulting in an immediate cessation of growth and glycerol consumption [14]. High level of 3-HPA also inhibit the activity of GDH [15] and can explain the low ratio of GDH and PDOR activities [16]. Not only the imbalanced activities of GDH and PDOR but also the high reversibility of PDOR result in the accumulation of 3-HPA. This leads to cellular toxicity, plasmid instability and the low production...
rate of 1,3-PD [17]. PDORI, encoded by yqhD, is also sufficient to catalyze the conversion of 3-HPA to 1,3-PD [18] and utilizes NADPH rather than NADH. This is more effective in 1,3-PD production than PDOR [19]. To improve 1,3-PD production, we propose to use a fusion protein of the related protein as a new approach for a more efficient coupling of the enzymes needed for 1,3-PD production. When protein DHAB is fused to YQHD, the occurrence of 3-HPA that is converted from glycerol by DHAB should be immediately reduced to 1,3-PD by YQHD. This strategy would be a possible way to solve the 3-HPA accumulation and also increase the rate of 1,3-PD production. In this paper, the structure of the fusion of YQHD and DhaB1 was investigated to consider the possibility for enhancing 1,3-PD production. After prediction of protein structure, we cloned and fused the following genes together: (1) yqhD from E. coli/BL21(DE3) encoding NADP-dependent 1,3-propanediol oxidoreductase isoenzyme (PDORI); and (2) dhaB1 and dhaB2 from C. butyricum VPI1718 encoding the coenzyme B12-independent glycerol dehydratase (GDH) and its activator (GDHtAc), respectively. This is the first study to use gene fusion for the production of 1,3-PD from glycerol.

C. DNA Manipulations

General procedures for DNA manipulations were performed according to Sambrook and Russel [23]. Genomic DNA of C. butyricum VPI1718 was extracted with a Genomic DNA Purification kit (Fermentas, Germany). Plasmid DNA was isolated by using NucleoSpin Plasmid Kit (Macherey-Nagel, Germany). DNA fragments were recovered from gels with NucleoSpin Extract II kit (Macherey-Nagel, Germany). Pfu DNA-polymerase and restriction enzymes (Fermentas, Germany) were used as recommended by the suppliers. DNA sequencing was carried out by using the ABI PRISM Dye Terminator (Perkin-Elmer, Germany).

D. Amplification of dhaB1-dhaB2 and yqhD

Genomic DNA of C. butyricum VPI1718 was extracted by a Genomic DNA Purification kit (Fermentas, Germany) according to the manufacturer’s instructions. The PCR mixture to amplify dhaB1-dhaB2 containing 2 mM each of dNTPs, 10x Pfu DNA polymerase buffer with MgSO4, 50 µM forward primer and reverse primer (3_P5F-2_P2R, 4_P7F-2_P2R), 1 U of Pfu DNA Polymerase and 100 ng of genomic DNA. The reaction was carried out by using the Touchdown program as follows; 95 °C for 5.30 min, followed by 11 cycles of 95 °C for 1 min; 57 °C (the temperature was decreased by 1 °C every cycle until the touchdown temperature of 47 °C was reached) for 1 min; 72 °C for 5 min; and then followed by 25 cycles of 95 °C for 1 min, 47 °C for 1 min, 72 °C for 5 min (the time was increased by 5 sec every cycle) and then 72 °C for 15 min. Two primer pairs (3_P4F-3_P4R, 3_P4F-4_P6R) were used to amplify yqhD from genomic DNA of E. coli BL21 (DE3). The PCR mixture contained 2 mM each of dNTPs, 10x Pfu polymerase buffer with MgSO4, 50 µM forward and reverse primers, 1 U of Pfu DNA Polymerase and 100 ng of genomic DNA. The reaction was carried out by using the Touchdown program as follows; 95 °C for 5.30 min, followed by 11 cycles of 95 °C for 1 min; 63 °C (the temperature was decreased by 1 °C every cycle until the touchdown temperature of 53 °C was reached) for 1 min; 72 °C for 5 min; and followed by 25 cycles at 95 °C for 1 min, 53 °C for 1 min, 72 °C for 5 min (the time was increased by 5 sec every cycle), and then 72 °C for 15 min. The PCR products were visualized by agarose gel electrophoresis and purified to use for construction of recombinant plasmid.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
</tr>
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<tbody>
<tr>
<td>2_P2R-dhaB</td>
<td>ATATGTGACCTACTCAGCTCCAAATTGT</td>
</tr>
<tr>
<td>3_P5F-dhaB</td>
<td>ATATAGATCCAGGAGATATAATGATAAGTAAGAG</td>
</tr>
<tr>
<td>4_P7F-dhaB</td>
<td>CTGAAGATTCCATGATAAAGGATTTAG</td>
</tr>
<tr>
<td>3_P4F-yqhD</td>
<td>CTGAGATCCATGATAAGGAGATTTAG</td>
</tr>
<tr>
<td>3_P4R-yqhD</td>
<td>ATATAGATCCAGGAGATATAATGATAAGTAAGAG</td>
</tr>
<tr>
<td>4_P6R-yqhD</td>
<td>ATATAGATCCAGGAGATATAATGATAAGTAAGAG</td>
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E. Construction of dhaB1-dhaB2 and yqhD into the Plasmid

The purified DNA fragments of dhaB1-dhaB2 were digested with the specific restriction enzyme and ligated into the plasmid, which had been dephosphorylated and linearized with the corresponding enzymes. Then, the ligation mixtures were transformed into E.coli JM 109 as a cloning host cell according to the technical manual. The purified plasmids from the candidate transformants were digested with specific restriction enzyme to check the inserted DNA fragment. The purified DNA fragments of yqhD were digested with the specific restriction enzyme and ligated into the positive recombinant plasmid harboring dhaB1-dhaB2, which had been dephosphorylated and linearized with the corresponding enzymes. Then, the ligation mixtures were transformed into the cloning host cells. Positive recombinant plasmids harboring dhaB1-dhaB2 and yqhD were transformed into expression host cells to use for protein expression and 1,3-propanediol production.

F. Production of 1,3-Propanediol from Glycerol by Different Recombinant Strains

Recombinant strains were cultivated in Riesenberg medium (RB) [24] which is derived from a medium developed for high cell density cultivations of E.coli. Modified RB medium for aerobic growth phase was composed of (per liter) 7 g glycerol, 13.3 g KH₂PO₄, 4.0 g (NH₄)₂HPO₄, 1.7 g citric acid, 8.4 mg EDTA and trace element. The trace element contained (per liter), 2.5 mg CoCl₂·6H₂O, 15 mg MnCl₂·4H₂O, 1.5 mg CuCl₂·2H₂O, 3.0 mg H₂BO₃, 2.5 mg Na₂MoO₄·2H₂O, 13 mg Zn(CH₃COO)₂·2H₂O and 100 mg Fe(III)citrate. To prevent the precipitation in the initial medium, stock solution of 50 g/l MgSO₄·7H₂O was sterilized separately and mixed afterwards to a final concentration of 1.2 g/l. A stock solution of 1 g/l thiamine was sterilized by filtration and combined at a final concentration of 45 mg/l. The medium was supplemented with ampicillin (100 µg/ml) when necessary. The fermentation culture was performed in a shake flask by incubation for 12 h at 37°C, 150 rpm on rotary shaker. Whole cells were harvested by centrifugation at 6,000 rpm for 15 min and resuspended with the medium for an anaerobic production stage. This stage contained RB medium supplemented with 0.05 M di-Na-fumarate, 0.05 M K’-Na’-tartrate and 0.1 mM IPTG. The cultivation was performed in septum bottles, incubated at 37°C, 150 rpm on a rotary shaker for 24 h. Cell growth, 1,3-PD and other metabolite products were determined. The investigation was performed in duplicate.

G. Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was conducted on a 10% (v/w) polyacrylamide gel by the method of Laemmli [25]. The operation was performed on the Mini-Protein III Electrophoresis System (Bio-Rad, USA). Proteins on the gel were stained with 0.2% (w/v) Coomassie Brilliant Blue R-250. Marker proteins with molecular weights ranging from 10 to 220 kDa were used to estimate the molecular weight of proteins.

H. Biomass, Glycerol, and 1,3-Propanediol Concentration

The biomass concentration was determined by measuring optical density at 600 nm and dry cell weight (grams dry weight per liter). Glycerol, 1,3-PD and other metabolite products were determined by HPLC (Agilent 1200). The culture medium was centrifuged at 12,000 g for 15 min, then, the supernatant was filtered through a nitrocellulose membrane with 0.2-µm pores. The HPLC apparatus included: a quaternary pump; a manual injector; a refractive index detector; an online vacuum degasser; a thermostatted column compartment; an Aminex HPX-87H ion exclusion column (300 mm x 7.8 mm) (Bio-Rad, USA); and ChemStation Software.

Operation conditions were: 20 µl sample volume; 5 mM H₂SO₄ as a mobile phase; flow rate of 0.7 ml/min and a column temperature of 65°C.

III. RESULTS AND DISCUSSIONS

A. Homology Modeling Analysis

The secondary structure prediction of the fusion protein of YQHD-DhaB1 using SWISS-MODEL tools reveals 8 sheets, 5 beta alpha beta units, 3 beta hairpins, 20 strands and 56 helices (Fig. 1). The Ramachandran analysis was used for structure evaluation of the fused YQHD-DHAB1. It was found that 768 residues of the predicted structure were in the most favored regions (74.10%). Additional allowed regions had 211 residues (20.40%), generously allowed regions had 31 residues (3.00%), and disallowed regions had only 26 residues (2.50%) (Fig.2).

![Fig. 1. The secondary structure and model prediction of the fusion of YQHD-DHAB1 using SWISS-MODEL](image-url)
residues were plotted in the most favored region, so the YQHD-DHAB1 fusion structure could be strong stability in the cell.

B. The YQHD-DHAB1 Interaction Analysis


The YQHD-DHAB1 model illustrated that the binding sites were in 2 main core regions. The first region consisted of the amino acid residues 738-740 and the second region consisted of residues 810-812, and residue 815 respectively. The YQHD-DHAB1 structure prediction showed that a large number of helices segments was observed. The helices segments are related to the entropic cost that is associates with the stabilizing interactions. Even though the analysis results of YQHD-DHAB1 fusion structure indicated that the structure of YQHD was changed after fusion with DhaB1, the effects of conformation change of YQHD was still unknown. Thus, the mechanism of these conformations of its binding sites effects and the levels of enzyme activity in production of 1,3-PD are under investigation.

C. Preparation of dhaB1-dhaB2 from Clostridium butyricum VPI1718

In this study, two primer pairs (3_P5F-2_P2R, 4_P7F-2_P2R) were used to amplify dhaB1-dhaB2 from genomic DNA of C. butyricum VPI1718. PCR products of dhaB1-dhaB2_P3 and dhaB1-dhaB2_P4 at a size of approximately 3.2 kb were purified to prepare for construction into the plasmid.

D. Construction of dhaB1-dhaB2 into Expression Vector

Purified PCR products of dhaB1-dhaB2_P3 and dhaB1-dhaB2_P4 were digested with BamHI and ligated into pET22b+(Novagen), which had been dephosphorylated and linearized with the corresponding enzymes. The ligation mixture of each system was transformed into E. coli JM109 according to the manufacturer’s instructions (Promega). The transformants of each system were selected on LB agar plate supplemented with 100 μg/ml ampicillin. The recombinant plasmids were purified and double digested with BamHI and SalI to confirm that the plasmids harboring the target fragment of dhaB1-dhaB2 was approximately 3.2 kb in size. Purified plasmid P31 and P41 containing pET22b+ harboring dhaB1-dhaB2_P3 and dhaB1-dhaB2_P4 were subjected to sequencing to confirm the nucleotide sequence of the inserted fragment.

E. Construction of yqhD into Recombinant Plasmid

Two primer pairs, 3_P4F-3_P4R and 4_P6F-4_P6R, were used to amplify yqhD from genomic DNA of E.coliBL21 (DE3) to yield of yqhD3 and yqhD4, respectively. The approximately 1.2 kb fragment of PCR products were purified to prepare for construction into the recombinant plasmid P31 and P41 that harboring dhaB1-dhaB2. Purified PCR product of yqhD3 and yqhD4 were double digested with Ncol-BamHI and ligated into P31 and P41, respectively, which had been dephosphorylated and linearized with the corresponding enzymes. The ligation mixture of each system was transformed into E. coli JM109 according to the manufacturer’s instructions (Promega). The transformants were selected on LB agar plate supplemented with 100 μg/ml ampicillin. The recombinant plasmid P31Y2 and P41Y3 were purified and triple digested with Ncol, BamHI and SalI to confirm that the plasmids harboring the target fragment of dhaB1-dhaB2 and yqhD were approximately 3.2 and 1.2 kb in size, respectively. Purified plasmid P31Y2 and P41Y3 were subjected to sequencing to confirm the nucleotide sequence of the inserted fragment. A representation of the recombinant

Fig. 3. Recombinant plasmids P31Y2 and P41Y3
plasmid P31Y2 and P41Y3 harboring engineered 1,3-PD operon is shown in Fig. 3.

In the first system, the recombinant plasmid P31Y2, the yqhD fragment was placed downstream of ribosome binding site in plasmid pET22b+. Downstream of yqhD, dhaB1 was immediately followed by dhaB2. A synthesized ribosome binding site (AAGGAG) was located on 5 bp upstream of the dhaB1 ATG initiation codon while dhaB2 was being preceded by a putative ribosome binding site (AGGGGA). This was located 8 bp upstream of the dhaB2 ATG initiation codon. In the second system, the recombinant plasmid P41Y3 contained the yqhD fragment, in which the stop codon (TAA) had been removed, followed by the dhaB fragment. The dhaB fragment contained the dhaB1 open reading frames fragment and the dhaB2 fragment including putative ribosome binding site. The yqhD was placed downstream of the T7 promoter in plasmid pET22b+. All of recombinant genes were transcribed in the same direction.

![SDS-PAGE analysis of expressed recombinant proteins](image_url)

**Fig.4.** SDS-PAGE analysis of expressed recombinant proteins when induced with a final concentration of 0.1 mM IPTG. Lane 1: insoluble proteins in BP; Lane 2: soluble proteins in BP; Lane 3: soluble proteins in E.coli BL21(DE3)/pET22b+yqhD dhaB1 dhaB2 (P31Y2); Lane 4: insoluble proteins in BP31Y2; Lane 5: soluble proteins in E.coli BL21(DE3)/pET22b+yqhD dhaB1 dhaB2 (P41Y3); Lane 6: insoluble proteins in BP41Y3. Expressed YQHD-DhaB1, DhaB1, DhaB2 and YQHD were indicated by the arrows.

**F. Protein Expression of Recombinant Strains**

The plasmid pET22b+, P31Y2 and P41Y3 were transformed into E.coliBL21 (DE3) as an expression host cell for protein expression. This yielded the recombinant E.coliBL21(DE3)/pET22b+(BP), E.coliBL21(DE3)/pET22b+yqhD dhaB1 dhaB2 (BP31Y2) and E.coliBL21(DE3)/pET22b+yqhD dhaB1 dhaB2 (BP41Y3), respectively. The protein expression was performed under aerobic conditions in LB medium supplemented with 100 μg/ml ampicillin and induced with a final concentration of 0.1 mM IPTG. Individual protein expression was achieved in the strain BP31Y2 while fusion protein expression was achieved in the strain BP41Y3. The expression of YQHD from strain BP31Y2 was visualized clearly on SDS-PAGE with the predicted molecular mass of 42 kDa, 88 kDa and 34 kDa, respectively (Fig. 4 Lane 4). In the case of strain BP41Y3, the fusion protein expression of YQHD and DHA1 was also clearly observed with the predicted molecular mass of 130 kDa which was composed of 42 kDa of YQHD and 88 kDa of DHA1. DHA2 was also visualized on SDS-PAGE as the strain BP31Y2 with the predicted molecular mass of 34 kDa (Fig. 4 Lane 6). All of the recombinant proteins could be expressed and visualized clearly on SDS-PAGE. This meant the translation processes of all recombinant proteins were successful. However, it was found that all recombinant proteins were inclusion bodies. Fenghua et al. [12] also reported that the expressed PDOR from E.coliBL21(DE3)/pET22b+-dhaT) were almost all inclusion bodies as well when it was performed under 37 °C. Although the pET22b+ contained pel signal peptide, it did not facilitate the secretion of recombinant PDOR into periplasm or could not fold the protein correctly in cytoplasm. Moreover, they reported that the induction of E.coliBL21(DE3)/pET28a+-dhaT) at 20°C could express the recombinant PDOR soluble in cytoplasm. This gave almost 3.3 times higher enzyme activity as compared with the condition at 37°C. The low temperature could decrease the protein synthesis and tended to increase the target protein in soluble form. This implies that temperature is a key factor for expressing the target protein in soluble form. Optimum conditions for expression of the recombinant proteins in soluble form are under investigation.

![Cell growth of the recombinant E.coliBL21(DE3)/pET22b+yqhD dhaB1 dhaB2 (P31Y2) and E.coliBL21(DE3)/pET22b+yqhD dhaB1 dhaB2 (P41Y3) under anaerobic production stage at 37°C.](image_url)

**Fig.5.** Cell growth of the recombinant E.coliBL21(DE3)/pET22b+yqhD dhaB1 dhaB2 (P31Y2) (▲) and E.coliBL21(DE3)/pET22b+yqhD dhaB1 dhaB2 (P41Y3) (■) under anaerobic production stage at 37°C. The high cell density inoculum was harvested from the aerobic growth stage.

**G. Production of 1,3-Propanediol from Glycerol by Different Recombinant Strains**

By fermentation of glycerol, the strain BP31Y2 and the strain BP41Y3 were investigated in RB medium under two-step conditions. Glycerol concentration of 7 g/l was used in the production stage medium instead of 30 g/l to avoid inactivation of GDH by glycerol [26]. High cell density of each strain was obtained under aerobic cultivation after 12 h. There was no production of 1,3-PD in this step. Then, each culture was harvested and resuspended to get an initial OD<sub>600</sub> of approximately 75 and transferred to the medium for...
anaerobic production stage. The cultivation was performed under anaerobic conditions at 37°C for 24 h. The cell growths of both strains in production stage are shown in Fig. 5. Initially, the cell growths of both strains increased slightly, and then declined in 2 h. As expected, the strain BP41Y3, YQHD and DHB1 expressed in form of fusion protein would convert 3-HPA to 1,3-PD immediately, hence, 3-HPA should not be accumulated in this system. However, it seems that 3-HPA still appeared during cultivation, resulting in a decrease of cell growth. The reason for 3-HPA accumulation may be presumably because of: (1) the unbalanced ratio of GDHt and PDOR activities [15]; and (2) the inadequate amount of reducing equivalents available from the oxidative pathway of glycerol as a cofactor for PDOR/PDORI activities.

Regarding the 1,3-PD production, the strain BP41Y3 was markedly different to strain BP31Y2 by giving 10-fold 1,3-PD concentration (Fig. 6). The final concentration of 1,3-PD and molar yield were 12.5 mmol/l and 0.23 mol/mol, respectively. Succinate, lactate and acetate were the by-products (data not shown). Nevertheless, strain BP41Y3 produced 1,3-PD quickly at an early cultivation time (2-8 h) but it kept constant later. This might be due to the occurrence of 3-HPA which has an inhibitory effect on GDHt and PDOR/PDORI activities [27]. At a high level of 3-HPA, it not only decreases the GDHt activity, which entails lower glycerol consumption [15], but also the PDOR/PDORI, which entails 3-HPA accumulation. Consequently, avoiding 3-HPA accumulation could be achieved by either the reduction of GDHt activity or the increment of PDOR/PDORI activities.

VI. CONCLUSION

Molecular modeling analysis was used to study the structure of a fusion protein of YQHD and DHB1 and to consider its possibility for 1,3-PD production. The results showed that although the conformation of YQHD was changed after fusion with DHB1, its change did not affect the binding sites. Therefore, it was possible to use this strategy for production of 1,3-PD. The expressed recombinant proteins from *E.coli*BL21(DE3)/pET22b+: yqhD-dhaB1_dhaB2 (strain BP31Y2) expressing the individual proteins of YQHD, DHB1 and DHB2 and *E.coli*BL21 (DE3)/pET22b+:: yqhD-dhaB1_dhaB2 (strain BP41Y3) expressing the fusion protein were all found to be inclusion bodies in the cells. By comparing 1,3-PD production, the strain BP41Y3 gave a 10-fold higher 1,3-PD concentration than the strain BP31Y2. This is the first report showing 1,3-PD production from glycerol using a gene fusion approach. Based on the results in the present study, further studies will involve the solving of inclusion bodies and elimination of 3-HPA accumulation during cultivation. In addition, the cultivation conditions and medium should be optimized to improve the 1,3-PD yield.

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