

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* VPI1718 coding for glycerol dehydratase (GDHt) and its activator (GDHtAc), 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 YQHD and DHAB1 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+::*yqhD-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.

R. R. is with the Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University, Hat-Yai, 90112 Thailand (email: rosarin_psu@yahoo.com).

P.P. is with the Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University, Hat-Yai, 90112 Thailand (phone:006674286369; fax: 006674446727; email: poonsuk918@yahoo.com).

A.P. is with the Center for Genomics and Bioinformatics Research, Faculty of Science, Prince of Songkla University, Hat-Yai, 90112 Thailand (email: pamornra@yahoo.com).

T.P. is with the Center for Genomics and Bioinformatics Research, Faculty of Science, Prince of Songkla University, Hat-Yai, 90112 Thailand (email: tanatepanrat@gmail.com).

J.S. is with the Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, 32 XiQiDao, Tianjin Airport Economic Area, Tianjin 300308, P. R. China and the Institute of Bioprocess and Biosystems Engineering, Hamburg University of Technology, Denickestrasse 15, Hamburg, 21073 Germany (email: sun.jibin@gmail.com).

S.R. is with the Institute of Bioprocess and Biosystems Engineering, Hamburg University of Technology, Denickestrasse 15, Hamburg, 21073 Germany (email: rappert@tu-harburg.de).

A.-P. Z. is with the Institute of Bioprocess and Biosystems Engineering, Hamburg University of Technology, Denickestrasse 15, Hamburg, 21073 Germany (email: aze@tu-harburg.de).

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) (PTT). This has superior properties such as good light stability, biodegradability and improved elasticity for the fiber industry [1], [2]. Over 10⁵ 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 microorganism 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 (GDHt), 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 GDHt 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 low 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 GDHt [15] and can explain the low ratio of GDHt and PDOR activities [16]. Not only the imbalanced activities of GDHt 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 (GDHt) and its activator (GDHtAc), respectively. This is the first study to use gene fusion for the production of 1,3-PD from glycerol.

II. MATERIALS AND METHODS

A. Homology Modeling Analysis

Homology modeling analysis was used to the two-dimensional (2D) and three-dimensional (3D) protein structure, protein conformations, and relative functions of unknown protein structures on the PDB database.

The protein homology modeling of the interested proteins was analyzed by using the servers available servers. One was SWISS-MODEL (<http://swissmodel.expasy.org>) [20] in which the best template was automatically selected based on multiple-threading alignments simulation. It was used for the structure prediction model. Next, each of the prediction models was validated on the best selected template by the PROCHECK software [21]. Finally, the ProFunc server (<http://www.ebi.ac.uk/thornton-srv/databases/profunc>) [22] was used to identify the functions of a protein using 3D protein structure.

B. Bacterial Strains, Plasmids, and Growth Conditions

C. butyricum VPI1718 was used for amplification of *dhaB1* and *dhaB2* encoding coenzyme B₁₂-independent glycerol dehydratase (GDHt) and glycerol dehydratase activator (GDHtAc), respectively. *E.coli* BL21(DE3) was used for amplification of *yqhD* encoding 1,3-propanediol oxidoreductase isoenzyme, NADP-dependent dehydrogenase, (PDORI), and used as a host cell for protein expression.

The plasmid pET22b+ (Invitrogen, Germany) was used for construction of the 1,3-propanediol operon. All the PCR primers were listed in Table I.

C. butyricum VPI1718 was precultured and cultured in 2X YT medium containing (per liter) 10 g yeast extract, 16 g Bactotryptone, 4 g NaCl and supplemented with 2% glucose.

Cultivation was incubated at 37°C, 16 h under anaerobic conditions for DNA extraction.

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 I
PCR PRIMERS FOR THE CONSTRUCTION OF RECOMBINANT PLASMIDS

Primer	Sequence (5' to 3')
2_P2R- <i>dhaB</i>	ATATGTCGACTTACTCAGCTCCAATGT
3_P5F- <i>dhaB</i>	ATTAGGATCCAAGGAGATATAATGATAAGTAAAG
4_P7F- <i>dhaB</i>	CTGAGGATCCATGATAAGTAAAGGATTAG
3_P4F- <i>yqhD</i>	CTGACCATGGATATGAACAACCTTAATCTGCACCC
3_P4R- <i>yqhD</i>	ATATGGATCCTTAGCGGGCGGCTTCGTATAT
4_P6R- <i>yqhD</i>	ATATGGATCCGCGGGCGGCTTCGTATAT

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 KH_2PO_4 , 4.0 g $(\text{NH}_4)_2\text{HPO}_4$, 1.7 g citric acid, 8.4 mg EDTA and trace element. The trace element contained (per liter), 2.5 mg $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 15 mg $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 1.5 mg $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 3.0 mg H_3BO_3 , 2.5 mg $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 13 mg $\text{Zn}(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$ and 100 mg Fe(III)citrate. To prevent the precipitation in the initial medium, stock solution of 50 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 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 $\mu\text{g}/\text{ml}$) 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 $\text{K}^+\text{-Na}^+$ -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 H_2SO_4 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).

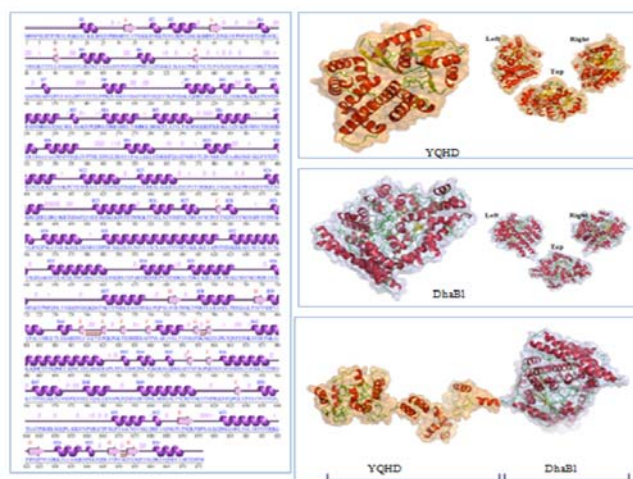


Fig. 1. The secondary structure and model prediction of the fusion of YQHD-DHAB1 using SWISS-MODEL

The Ramachandran analysis results showed the quality of the YQHD-DHAB1 fusion structure in the cell. It revealed that the residue elements from the final prediction structure model of the fused YQHD-DHAB1 were plotted in the allowed regions (>95%), especially the 74% of the amino acid

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.

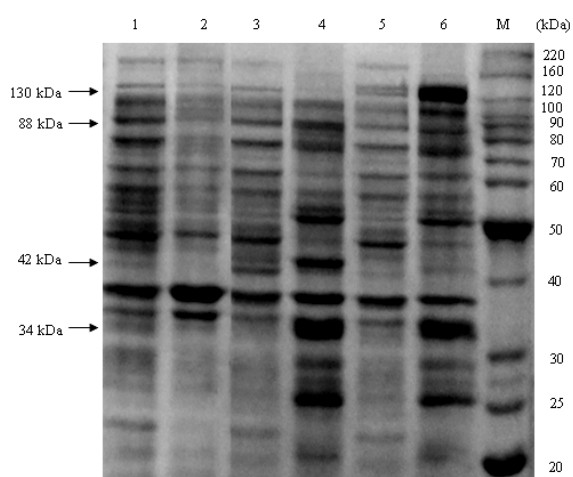


Fig.4. SDS-PAGE analysis of expressed recombinant proteins when induced at 37°C. Lane M: protein molecular weight marker; Lane 1: soluble proteins in *E.coliBL21(DE3)//pET22b+* (BP); Lane 2: insoluble proteins in BP; Lane 3: soluble proteins in *E.coliBL21(DE3)//pET22b+-yqhD_dhaB1_dhaB2* (BP31Y2); Lane 4: insoluble proteins in BP31Y2; Lane 5: soluble proteins in *E.coliBL21(DE3)//pET22b+-yqhD-dhaB1_dhaB2* (BP41Y3); 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 DHAB1 was also clearly observed with the predicted molecular mass of 130 kDa which was composed of 42 kDa of YQHD and 88 kDa of DHAB1. DHAB2 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. Fenghaun *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.

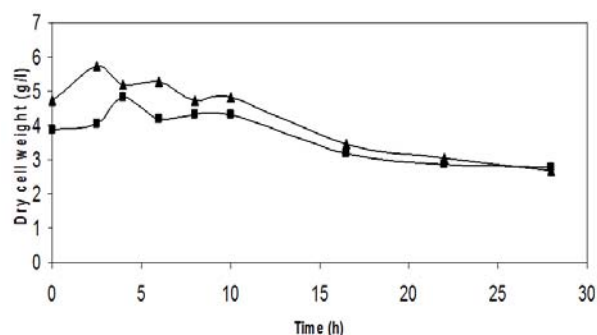


Fig.5. Cell growth of the recombinant *E.coliBL21(DE3)//pET22b+-yqhD_dhaB1_dhaB2* (P31Y2) (▲) and *E.coliBL21(DE3)//pET22b+-yqhD-dhaB1_dhaB2* (P41Y3) (■) under anaerobic production stage at 37°C. The high cell density inoculum was harvested from the aerobic growth stage.

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 GDHt 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 OD₆₀₀ 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 DHAB1 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 activity.

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.

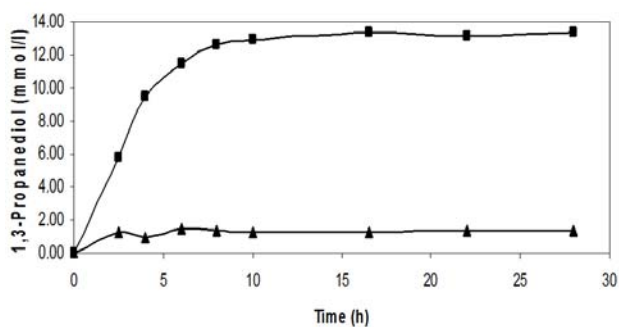


Fig.6. 1,3-Propanediol formation by the recombinant *E.coli*BL21(DE3)//pET22b+ *yqhD_dhaB1_dhaB2* (P31Y2) (▲) and *E.coli*BL21(DE3)//pET22b+*-yqhD-dhaB1_dhaB2* (P41Y3) (■) under anaerobic production stage at 37°C

VI. CONCLUSION

Molecular modeling analysis was used to predict the structure of a fusion protein of YQHD and DHAB1 and to consider its possibility for 1,3-PD production. The results showed that although the conformation of YQHD was changed after fusion with DHAB1, 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,

DHAB1 and DHAB2 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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