NICVB Journal of Science Control Vaccines and Biologicals, Vol.4, No.3 (2024)

EXPRESSION ENZYME CONVERT GLYCEROL TO DIHYDROACETONE FROM BACTERIA

Nguyen Quyet Thang[1*] , Gyeong Soo Ko[2]*, Doan Huu Thien[1], Duong Thi Cam Le[1], Dam Thi Lieu[1], Pham Quang Minh[1], Canh Huyen Trang[1], Bui Thanh Tung[1], Kang Wonchull[2], Jang Jin Kuk[2].

[1]National Institute For Control of Vaccines and Biologicals

[2]Soongsil University, South Korea

Received 06 May 2024

Accepted 30 August 2024

Abstract: Glycerol dehydrogenase plays an important role in the glycerol metabolic pathway. During oxidation, glycerol is converted to dihydroxyacetone (DHA), and it is the first step in this cycle. Nowadays, DHA is synthesized because of its value and application in many industries. In this study, we focused on expressing the enzyme capable of converting glycerol into DHA on the bacteria strain *Klebsiella pneumoniae*. The results of gene expression and purification of recombinant protein encoding the enzyme GlyDH are presented in this study. With an in vitro cell culture volume of 1 liter and high expression and purification capabilities, it shows great potential in practical application.

Keywords: Gene expression, protein purification, bacterial enzyme,…

1. Introduction

Glycerol dehydrogenase (GlyDH, E.C.:1.1.1.6) is an enzyme that converts glycerol to dihydroxyacetone (DHA) by using NAD^+ (Nicotinamide adenine dinucleotide) as a cofactor[1].

GlyDH also plays an important role in converting acetone into 2,3-butanediol in in-vivo [2]. It is not only an important enzyme in the metabolism pathway but also very valuable in industries. Such as DHA 1,3-Dihydroxy-2-propanone (CAS No. 96- 26-4, EC. No 202-494-5) from GlyDH is a chemical widely used in chemistry, especially in cosmetics applicable as a toner, in aging

* Corresponding author
E-mail address: Thang50cnsh@gmail.com, gyeongsooko@gmail.com
https://doi.org/10.56086/jcvb.v4i3.163

and whitening, and approved by FDA or other names are dihyxal, otan, oxantin.

In the food industry, GlyDH is also widely applicable from a chemical to synthesize and produce antifreeze fluids such as 1,2-propylene glycol to countless bioactive compounds used in pharmaceuticals, pesticides, and sweeteners. [3-5]. Additionally, GlyDH is used in the conversion of biofuel products such as ethanol, 1,3-propanediol and 2,3-butanediol [6-8].

Glycerol-metabolizing enzymes are expressed in a wide range of organisms, including bacteria, yeasts, and mammals. These enzymes are divided into three main groups, depending on the site of oxidation and the substrate involved in the reaction. Under anaerobic conditions, many microorganisms utilize glycerol as a carbon substrate by both oxidative and reductive pathways.

However, our group is the first group studies have been conducted on the *Klebsiella pneumoniae strain*. *The Klebsiella pneumoniae strain* was chosen as a subject because this train has two genes encoding for GlyDH (*dhaD* and *gldA*). This is very different from other bacteria. In this study,

Use the primers as we described above to run PCR by following principles:

GlyDH is encoded *gldA* gene from *K. pneumoniae* MGH78578 to use for expression and purification.

Therefore, the expression and purification of glycerol dehydrogenase are very widely applied in Vietnam.

2. Materials and method

2.1. Materials

The genomic DNA of *K. Pneumoniae strain* MGH78578 (ATCC 700721) was supplied by ATCC (USA).

- PCR kit and Plasmid kit from Geneall (Korea).
- *E.coli strain* DH10B was used for cloning, and the Rosetta 2 strain (DE3) was used for expression.
- Cultivation using LB medium adding antibiotic Kanamycin.

2.2. Method

2.2.1. Primers

The gene encoding for enzyme *K. pneumoniae strain* MGH78578 was used as a template. These primer sequences were: 5‘-AGCGACGCATATGGATCGCATTATTCAA-3’ “forward” and 5’CTGTTCTCGAGTTCCCACTCTTGAAGGAAGCGCTGACC-3’ “reverse”.

2.2.2. PCR condition (Polymerase chain reaction)

Temperature (^{0}C)	**Time (Second)**	**Cycles**

50	120	1
95	120	1
95	15	Repeat 35 times
60	60	
Stay at 4 ^{0}C until shutdown		

2.2.3. Plasmid

PCR products were treated with restriction enzyme NdeI và XhoI, after that inserted into the pET26b vector (also treated with the same restriction enzyme), These His6-tags were added at the C-terminal of the protein by insert gene. Then the insert vector was transferred into *E.coli* DH10B, The colonies were selected and cultured in LB agar adding 30 µg/ml antibiotic Kanamycin.

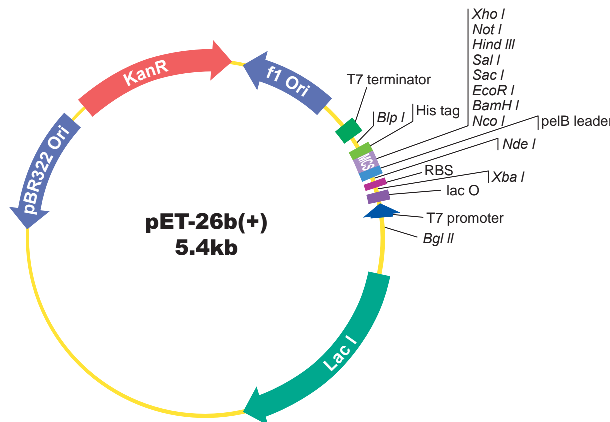

Figure 1. pET26b plasmid with restriction enzyme map. ***Source: genscript.com***

2.2.4. Sequencing

- The sequencing of these nucleotides of strains was analyzed and confirmed by Bionics Korea. All operations performed on DNA such as gene isolation and plasmid collection follow the instructions of Geneall Kit "PCR kit và Plasmid kit".

- Gene bank NCBI was use to check the sequencing data (https://www.ncbi.nlm.nih.gov/genbank/). These amino acid information were compared in Uniprot data bank with code A6TGD6 (https://www.uniprot.org/uniprot/A6TGD6).

2.2.5. Protein expression

- After confirming the sequence, select to culture insert plasmid by transfer in Rosetta 2 (DE3) cells.

- Gene expression test was performed by culturing the strain on Rosetta 2 (DE3) cells in 10 ml LB medium containing 30 µg/ml

Kanamycin antibiotic at 37^0C, shaking in 12-16 hours. The transfer 1 ml cell culture to 1 litter of LB medium adding 30 µg/ml Kanamycin antibiotic and cell culture at different temperatures 37^0C, 25^0C và 20^0C to check the expression level (follow the instruction of the company for BL21 "DE3" strain).

- At 37^0C: cell culture until OD_{600} reach to 0,5-0,6; adding IPTG (Isopropyl ß-D-1-thiogalactopyranoside) to activate the protein expression. After that, continuous cultivation was performed for 4 hours, and cells were collected.

- At 25^0C và 20^0C cell culture at 37^0C until the OD_{600} reached 0,5-0,6; quickly move to the ice box to fast cooling in 15 minutes. After that, adding the IPTG to activated, and continuous cultivation at 25^0C and 20^0C, continuous shaking for 12 hours and collecting of the cells.

- Collecting cells by centrifuge the cell culture at 15000 rpm/min at 15^0C in 30 minutes. Discard the supernatant and reconstitute the precipitate by adding buffer solution containing 20 mM Tris-HCl, 0.1 mM TCEP, 5% ethylene glycol, 100 mM NaCl, and 10 mM imidazole, pH 8.0.

2.2.6. Gene expression evaluation

Gene expression result was evaluated through the results of the SDS-Page experiment of I-, I_{+1}, I_{+4}, I_{ON}, Cell, and Sup samples.

- Collection 1 ml cell culture at a different temperature at the time before adding IPTG (I-), after adding IPTG 1 (I_{+1}) hours, after adding IPTG 4 hours (I_{+4}) and overnight with the cell culture at 25^0C và 20^0C (I_{ON}), transfer into eppendorf 1,5 ml and then centrifuged to collect the cell and prepare to run the SDA-PAGE.

- The cell after reconstitution extracted the protein using a sonicator, after beak the cell, was centrifuged at 12000 rpm/min at 15^0C for 30 minutes. Carefully collect the supernatant "Sup". The cells after centrifuge "Pellet" were washed 3-4 times with distilled water and reconstituted by NaCl 0,9% "Cell".

- Run the SDS-PAGE with the samples following the order I-; I_{+1}; I_{+4}; I_{ON}; Sup; and Cell. Based on the SDS-PAGE results, evaluate the expression at different cultivation temperatures.

2.2.7. Protein Purification

- As the result of protein expression optimize the expression condition. We culture 1 litter to test the expression and purification of protein.

- The principle was the same as we described above. After collecting the supernatant, the Affinity Chromatography column was used to purify in the first step

using the His-Tag column. Washing and collecting the protein by increasing the Immidazole concentration (gradient) of the mobile phase. Run SDS-PAGE to check the result of the purification step.

3. Results

3.1. Isolations and insert gene

- The result of agarose gel Figure 2 shows that the position S6 và S12 in agarose, two bands appear compared to the standard ladder 1000 bp/1kb.
- Sequencing results of insert plasmid were compared with data bank by Blast tool. The Results were a complete match to the *gldA gene* from *K. Pneumoniae strain code* MGH78578 (Figure 3).

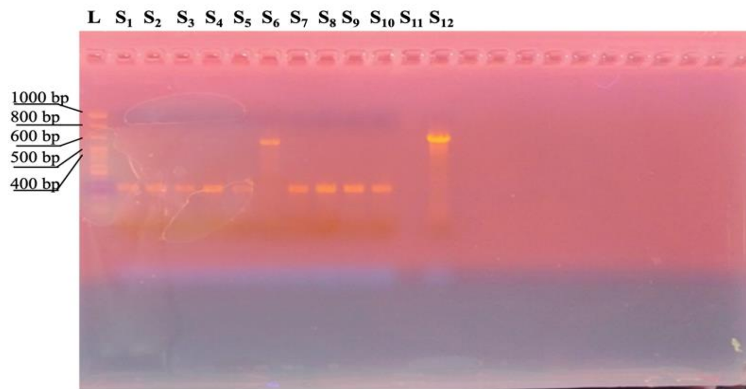

Figure 2. Electrophoresis results of inserting plasmid.

```
BLASTN 2.5.0+

Reference:
Zheng Zhang, Scott Schwartz, Lukas Wagner, and Webb Miller (2000),
"A greedy algorithm for aligning DNA sequences", J Comput Biol 2000;
7(1-2):203-14.


Database: Nucleotide collection (nt)
           41,670,128 sequences; 140,620,651,272 total letters


Query= 022618-BA-001-1-kp1-T7

Length=1396
                                                                      Score     E
Sequences producing significant alignments:                          (Bits)  Value

CP000647.1  Klebsiella pneumoniae subsp. pneumoniae MGH 78578, co...  1969    0.0
CP017985.1  Klebsiella pneumoniae strain 825795-1, complete genome    1958    0.0
CP015382.1  Klebsiella pneumoniae strain CN1, complete genome         1958    0.0
CP018719.1  Klebsiella pneumoniae strain KP_Goe_828304, complete ...  1958    0.0
CP018713.1  Klebsiella pneumoniae strain Kp_Goe_152021, complete ...  1958    0.0
CP018707.1  Klebsiella pneumoniae strain Kp_Goe_827026, complete ...  1958    0.0
CP018701.1  Klebsiella pneumoniae strain Kp_Goe_827024, complete ...  1958    0.0
CP018695.1  Klebsiella pneumoniae strain Kp_Goe_149832, complete ...  1958    0.0
CP018686.1  Klebsiella pneumoniae strain Kp_Goe_149473, complete ...  1958    0.0
CP018140.1  Klebsiella pneumoniae strain Kp_Goe_822579, complete ...  1958    0.0
CP012426.1  Klebsiella pneumoniae strain KP5, complete genome         1958    0.0
CP012745.1  Klebsiella pneumoniae subsp. pneumoniae strain TGH13,...  1958    0.0
CP015025.1  Klebsiella pneumoniae strain Kpn223, complete genome      1958    0.0
CP014755.1  Klebsiella pneumoniae strain AATZP, complete genome       1958    0.0
CP015500.1  Klebsiella pneumoniae strain SKGH01, complete genome      1958    0.0
LN824133.1  Klebsiella pneumoniae genome assembly MS6671.v1, chro...  1958    0.0
```

Figure 3. Blast result of sequencing

The data from blast is verify the gene Kết quả cho thấy trình tự so sánh hoàn toàn trùng khớp với chủng *Klebsiella pneumoniae,* hay trình tự gen mã hoá cho enzyme GlyDH đã được nhân và chèn thành công vào trong plasmid pET26b.

3.2. Optimize expression condition

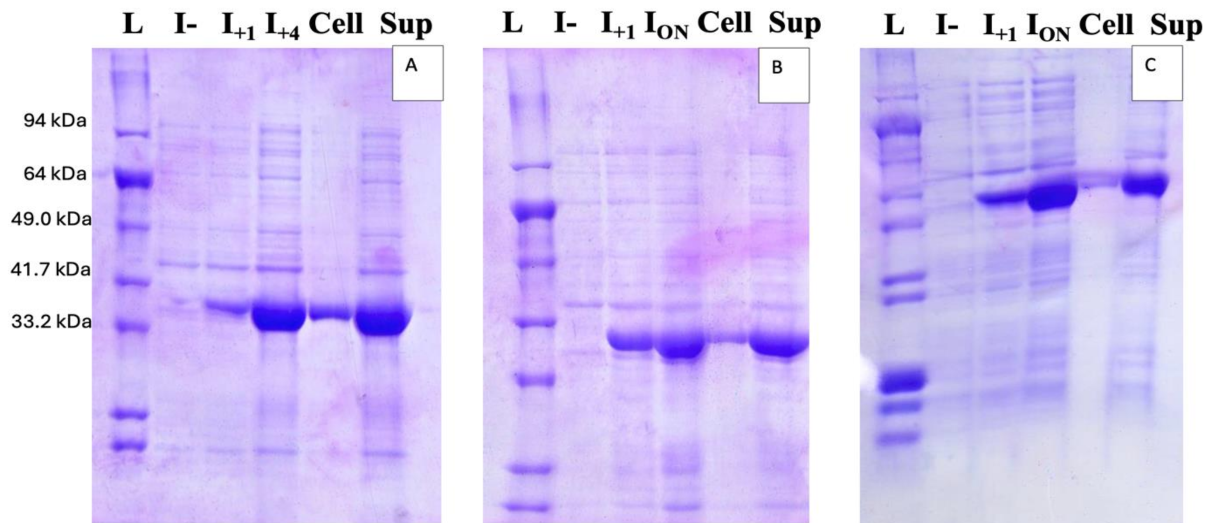

Figure 4. Protein expression at 37^0C (A), 25^0C (B) và 20^0C (C). Với I-; before adding IPTG, I_{+1}; after adding IPTG 1 hour, I_{+4}; after adding IPTG 4 hours, I_{ON}; Overnight, Sup; Supernatant và Cell; precipitation.

- The gene expression results were demonstrated by comparing the protein content obtained before and after IPTG addition, as well as comparing the gene expression results at different temperatures. Before gene expression activation, no band appeared at the molecular size position. 38.748,3 Da (between 41,7 kDa and 33,2 kDa), compared with the lines that appeared after adding the activator.

- The results in Figure 4. show that the gene can be expressed at all temperatures. At 20^0C (Figure 4.C), the protein content obtained is the highest and the protein solubility after expression is the best.

3.3 Enzyme purification result

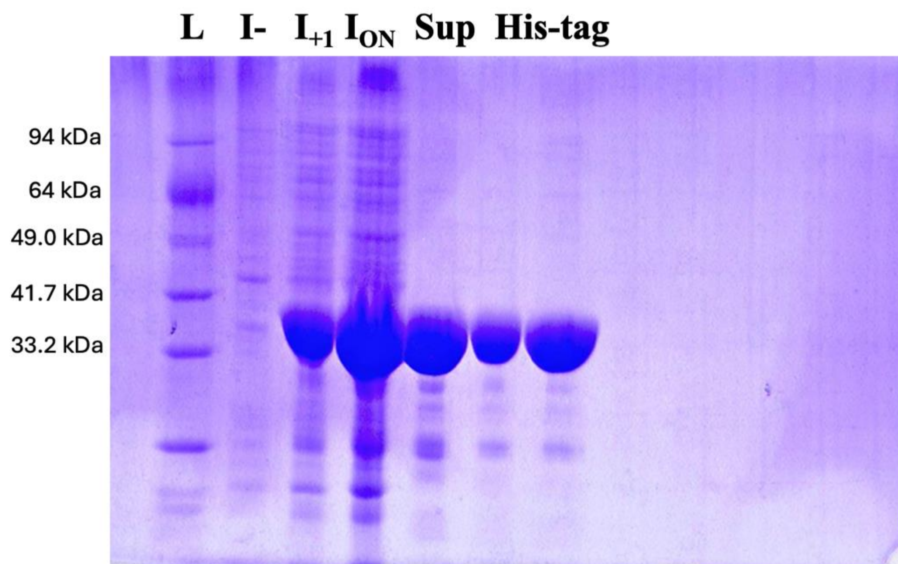

Figure 5. Results of large-volume protein cultivation and purification.

Based on the results of SDS-Page gel electrophoresis (Figure 5.), we can see that the recombinant protein is completely expressed and purified. The purity of the protein after using the His-tag chromatography column is over 90%.

4. Discussion

The process of isolating genes from *K. pneumoniae* through PCR reaction and transferring genes into plasmids has been successfully performed by us through electrophoresis and sequencing results. The electrophoresis image results with genes inserted into plasmids have sizes ranging

from 500 bp-600 bp, consistent with the size of the target gene. Through gene sequencing and comparison with the gene bank, the gene coding for the GlyDH enzyme was taken from *K. pneumoniae and* confirmed successful transfer into the plasmid using the pET26b vector.

After the gene transfer process, the expression process was performed by the culture method supplemented with an activation signal. With the temperatures studied, the results showed that the gene encoding the GlyDH enzyme was obtained from *K. pneumoniae* completely capable of being expressed easily and with high expression efficiency. Through the optimal culture conditions, we also selected the most suitable temperature to conduct the recombinant protein purification test after being expressed. The protein will be more stable and stable in the slow expression condition that prolongs the time of the logarithmic phase, by lowering the temperature and prolonging the gene expression time. At 37^0C (Figure 4.A), we can clearly see that after adding IPTG to activate gene expression, the gene expression efficiency as well as the ability to collect are poor, but due to the short expression time and this temperature is the optimal temperature for E.coli strain to grow, the growth time is shortened, causing the protein to be expressed but not completely (comparing the content of I_{Sup} và I_{+4}), at 20^0C almost all of the protein produced in the cell can be collected.

Using affinity chromatography (His-tag) showed that the purification process of recombinant protein encoding GlyDH enzyme achieved very high efficiency when comparing the I_{ON} sample and His-tag sample before and after the purification, based on the results of Figure 5.

5. Conclusion and recommendation

The above results demonstrated that we successfully expressed the gene encoding the GlyDH enzyme obtained from the bacteria *K. Pneumoniae strain*.

The recombinant protein purification results we presented also show that the fully expressed gene can be purified and obtained at high levels, and this research result can be used to develop future application studies.

After the purification process, to achieve higher concentration and purity, we propose in future studies to increase the culture volume and use additional purification steps after going through the His-tag affinity chromatography column such as ion exchange, and molecular size chromatography to achieve high-purity sample quality and bring better applicability.

References

[1] Ko GS, Nguyen QT, Kim DH, Yang JK. Biochemical and Molecular Characterization of Glycerol Dehydrogenase from *Klebsiella pneumoniae*. Journal of Microbiology and Biotechnology. 2020 Feb 28;30(2):271–8.

[2] Wang Y, Tao F, Xu P. Glycerol Dehydrogenase Plays a Dual Role in Glycerol Metabolism and 2,3-Butanediol Formation in *Klebsiella pneumoniae*. Journal of Biological Chemistry. 2014 Feb;289(9):6080–90.

[3] Hekmat D, Bauer R, Fricke J. Optimization of the microbial synthesis of dihydroxyacetone from glycerol with Gluconobacter oxydans. Bioprocess and Biosystems Engineering. 2003 Dec 1;26(2):109–16.

[4] Beauchamp J, Gross PG, Vieille C. Characterization of Thermotoga maritima glycerol dehydrogenase for the enzymatic production of dihydroxyacetone. Appl Microbiol Biotechnol. 2014 Aug;98(16):7039–50.

[5] Weckbecker A, Gröger H, Hummel W. Regenration of Nicotinamide Coenzymes: Principles and Applications for the Synthesis of Chiral Compounds. In: Wittmann C, Krull R, editors. Biosystems Engineering I [Internet]. Berlin, Heidelberg: Springer Berlin Heidelberg; 2010 [cited 2024 Feb 2]. p. 195–242. Available from: http://link.springer.com/10.1007/10_2009_55

[6] Tao F, Tai C, Liu Z, Wang A, Wang Y, Li L, et al. Genome Sequence of *Klebsiella pneumoniae* LZ, a Potential Platform Strain for 1,3-Propanediol Production. J Bacteriol. 2012 Aug 15;194(16):4457–8.

[7] Petrov K, Petrova P. High production of 2,3-butanediol from glycerol by *Klebsiella pneumoniae* G31. Appl Microbiol Biotechnol. 2009 Sep;84(4):659–65.

[8] Oh BR, Seo JW, Heo SY, Hong WK, Luo LH, Joe M ho, et al. Efficient production of ethanol from crude glycerol by a *Klebsiella pneumoniae* mutant strain. Bioresource Technology. 2011 Feb;102(4):3918–22.