

Cofactor Regeneration Using Permeabilized *Escherichia coli* Expressing NAD(P)⁺-Dependent Glycerol-3-Phosphate Dehydrogenase

Ho Sik Rho and Kyungoh Choi*

Department of Chemical and Material Engineering, The University of Suwon, Hwaseong 18323, Republic of Korea

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*Corresponding author
Phone: +82-31-229-8542;
Fax: +82-31-220-2528;
E-mail: kyungoh98@suwon.ac.kr

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Oxidoreductases are effective biocatalysts, but their practical use is limited by the need for large quantities of NAD(P)H. In this study, a whole-cell biocatalyst for NAD(P)H cofactor regeneration was developed using the economical substrate glycerol. This cofactor regeneration system employs permeabilized *Escherichia coli* cells in which the *glpD* and *gldA* genes were deleted and the *gpsA* gene, which encodes NAD(P)⁺-dependent glycerol-3-phosphate dehydrogenase, was overexpressed. These manipulations were applied to block a side reaction (*i.e.*, the conversion of glycerol to dihydroxyacetone) and to switch the *glpD*-encoding enzyme reaction to a *gpsA*-encoding enzyme reaction that generates both NADH and NADPH. We demonstrated the performance of the cofactor regeneration system using a lactate dehydrogenase reaction as a coupling reaction model. The developed biocatalyst involves an economical substrate, bifunctional regeneration of NAD(P)H, and simple reaction conditions as well as a stable environment for enzymes, and is thus applicable to a variety of oxidoreductase reactions requiring NAD(P)H regeneration.

Keywords: Cofactor regeneration, glycerol-3-phosphate dehydrogenase, NAD(P)H, permeabilized whole-cell biocatalyst

Introduction

Nicotinamide cofactor (NAD(P)H) regeneration remains an important issue in the efficiency of biotransformation, in which enzymes and microorganisms are used to synthesize various chemicals [1, 2]. Oxidoreductases have wide applications in industry-relevant reactions [3, 4]. However, these oxidoreductases usually require NAD(P)H, which is too expensive when stoichiometric amounts are used [5]. To overcome this limitation, several in situ cofactor regeneration methods have been developed [6, 7]. Among these approaches, the use of a second enzyme to regenerate cofactors by transforming a sacrificial substrate is particularly common. Enzymes used for the cofactor regeneration include formate dehydrogenase [8], alcohol dehydrogenase [9], glucose-6-phosphate dehydrogenase [10], glucose dehydrogenase [11], and phosphite dehydrogenase [12].

Several factors must be considered when developing an enzymatic regeneration system. The cost of the sacrificial substrate is an essential factor, as the necessary amount of

this substrate is equivalent to that of the desired conversion product. Additionally, an enzyme that regenerates both NADH and NADPH possesses a practical advantage over enzymes specific to a single cofactor. In some cases, the specificity of the cofactor has been modified by rational design [13], but modification may be difficult or laborious. Finally, stability and simplicity are also important for the development of cofactor regeneration methods. The utilization of permeabilized cells as an enzyme source is a promising way to achieve both stability and simplicity [14].

In this study, a novel cofactor regeneration system was developed using a permeabilized *Escherichia coli* whole-cell biocatalyst harboring an overexpressed glycerol-3-phosphate dehydrogenase (G3PDH). The main physiological function of G3PDH is the formation of glycerol-3-phosphate (G3P) from dihydroxyacetone phosphate (DHAP) and the consumption of NAD(P)H for the synthesis of phospholipids [15]. From the NAD(P)H-consuming reaction, a reverse reaction to produce NAD(P)H is expected to be feasible with an abundant G3P supply. As the G3P supply is

obtained from glycerol degradation pathways, NAD(P)H may be generated by the G3PDH reaction using the economical substrate glycerol. Recently, the growth of the biodiesel industry has led to an increased supply of glycerol, which is generated during biodiesel production [16]. As a consequence, the price of glycerol has fallen considerably [17]. To demonstrate the designed cofactor regeneration system, a coupling reaction, in which cofactors are consumed to generate products, was introduced. The coupling reaction employed in this work was a lactate dehydrogenase reaction in which pyruvate is transformed into lactate with the consumption of NADH. This coupling reaction was chosen owing to the simplicity of the colorimetric method for the detection of pyruvate concentration. The conversion efficiency of pyruvate was ~65%, whereas there was no conversion without the cofactor regeneration biocatalyst. The results showed that G3PDH in permeabilized *E. coli* cells could lead to cofactor regeneration utilizing economical glycerol.

Materials and Methods

Chemicals

α -Glycerophosphoric acid disodium salt was purchased from Tokyo Chemical Industry Corporation (Japan). DHAP and 2,4-dinitrophenylhydrazine (DNP) were purchased from Sigma-Aldrich (USA). Xylene was obtained from the Lancaster Synthesis Company (USA). All other materials were of analytical grade and commercially available.

Strains, Plasmids, and Enzymes

E. coli Tuner (Novagen, USA) and K-12 W3110 cells were used

as host strains for the G3PDH expression. The former was employed to measure in vitro G3PDH activity, and the latter was used for cofactor regeneration in the whole-cell catalyst. The plasmids pCDFDuet and pTrc99A were used as expression vectors. For the deletion of target genes, several plasmids (pKD46, pKD3, pKD4, and pCP20) were used [18]. All restriction enzymes and T4 ligase were purchased from New England Biolabs (USA) and Roche Applied Sciences (Germany), respectively. Lactate dehydrogenase for the coupling reaction, which was used to estimate the efficiency of the cofactor regeneration system, was purchased from Sigma-Aldrich (USA).

Construction of G3PDH-Overexpressing Plasmids

The genomic DNA of *E. coli* K-12 W3110 was used as a template, and the *gpsA* gene, which encodes G3PDH, was amplified by PCR with primers 1 and 2, listed in Table 1. Each primer contained EcoRI/AflIII restriction sites. The amplified gene fragments were cloned into the pCDFDuet and pTrc99A vectors treated with the same restriction enzymes to generate the vectors pCDF-gpsA and pTrc-gpsA, respectively.

Assay of G3PDH Activity

G3PDH activity was determined in Tuner cells carrying pCDF-gpsA. The transformant was cultivated at 37°C in Luria-Bertani (LB) medium with 50 μ g/ml spectinomycin. When the cell density reached an OD₆₀₀ of 0.6, 1 mM isopropyl β -D-thiogalactopyranoside (IPTG) was added to induce G3PDH expression. After 4 h of incubation, the cells were harvested by centrifugation (4,000 \times g, 15 min). Subsequently, 1 mg/ml lysozyme was added, and the cells were sonicated six times on ice for 10 sec each, at 5 sec intervals. Following centrifugation (10,000 \times g, 25 min) at 4°C, supernatants were collected. These supernatants were used to determine G3PDH activity. The oxidation of G3P with NAD⁺ or NADP⁺ is described as a NAD(P)H-generating reaction. G3PDH

Table 1. List of primers used for the PCR.

Primer	Nucleotide sequence	Target/Purpose
1	5'-GAATTCGATGAACCAACGTAATGCTTC-3'	N-terminus of G3PDH
2	5'-CTTAAGTTAGTGCTGCTGCGCTC-3'	C-terminus of G3PDH
3	5'-ATGGACCGCATTATCAATCACCGGTAAATACATC CAGGGCGCTGATGTGTAGGCTGGAGCTGCTTC-3'	Construction of <i>gldA</i> gene deletion cassette
4	5'-GTTATTCCTTCTTGCAGGAAACGCTGACCGTACTG GTCGGCTACCAGCCATATGAATATCCTCCTTAG-3'	
5	5'-CATTTATGAGCTTTAACGAAAGTGAATGAGGGCAGC ATGGAAACCAAAGGTGTAGGCTGGAGCTGCTTC-3'	Construction of <i>glpD</i> gene deletion cassette
6	5'-CAGGCCAGATTGAAATCTGACCTGATCACCTTACGTT AATTTACGACGCCATATGAATATCCTCCTTAG-3'	
7	5'-CTACTCATCTCTAAAGGAGC-3'	Verification of <i>gldA</i> gene deletion
8	5'-GGACAAGCCGGGAGTTTG-3'	
9	5'-CAATGTTACCTAAAGCGCGATTTC-3'	Verification of <i>glpD</i> gene deletion
10	5'-CCACGTAGGCCTGATAAGC-3'	

activity for the NAD(P)H-generating reaction was determined at room temperature with the following conditions: the 1 ml reaction mixture contained 50 mM potassium phosphate (pH 7.0) or carbonate (pH 9.0) buffer, 2 mM NAD⁺ or NADP⁺, 2 mM G3P, 100 mM hydrazine, and 10 μ l of Tuner (pCDF-gpsA) cell lysates. The reactions were initiated by the addition of the lysates, and activity was evaluated by measuring the absorbance at 340 nm, which was converted to the NAD(P)H concentration on the basis of NAD(P)H standard curves.

Construction and Permeabilization of the Cofactor Regeneration Strain

For the effective utilization of glycerol as a sacrificial substrate, the *gldA* and *glpD* genes were disrupted. Briefly, a linear DNA fragment containing 50 bp homologous to the upstream region of the target gene, a kanamycin or chloramphenicol resistance gene from a pKD4 or pKD3 plasmid, and 50 bp homologous to the downstream region of the target gene was obtained by PCR with primers 3–6, listed in Table 1. The 779 or 935 bp PCR product was transformed into *E. coli* K-12 W3110 carrying a pKD46 plasmid, and colonies containing homologous recombination products were selected by kanamycin or chloramphenicol resistance. Target gene disruption was confirmed by PCR with primers 7–10, listed in Table 1. In this manner, the W3110 mutant (W3110 Δ *glpD* Δ *gldA*) in which both *glpD* and *gldA* were deleted was constructed. Then, the pTrc-gpsA vector was transformed into these cells, and the obtained transformant was defined as the cofactor regeneration strain, designated W3110 Δ DA (pTrc-gpsA).

This strain was grown in 20 ml of LB medium supplemented with 0.5 mM IPTG at 37°C. The overnight culture was harvested by centrifugation and washed twice with 50 mM potassium phosphate buffer (pH 7.0). The washed cells were collected by centrifugation and resuspended to reach an OD₆₀₀ of 40. A 0.5 ml cell suspension was treated with 2% (v/v) xylene for 10 min. The permeabilized cells obtained in this manner were used for the cofactor regeneration.

Assay of Lactate Dehydrogenase Activity

Lactate dehydrogenase catalyzes a reaction in which pyruvate is converted to lactate using stoichiometric amounts of NADH. Therefore, the activity of lactate dehydrogenase may be determined by measuring the decreases in NADH or pyruvate concentrations. The 1 ml reaction mixture used for the assay contained 50 mM potassium phosphate buffer (pH 7.0), 0–3 mM NADH, 10 mM pyruvate, and 25 units of lactate dehydrogenase enzyme. The concentration of NADH was measured spectrophotometrically at 340 nm. Another approach to determine concentrations of pyruvate is to use a colorimetric assay utilizing DNP [19]. In detail, 0.2 ml of 1.25 mM DNP was added to the 10-fold-diluted reaction mixture, and the solution was incubated for 5 min. Subsequently, 0.8 ml of 1.5 M NaOH was added, and absorbance was measured at 445 nm after an additional incubation of 5 min. The absorbance was converted to the pyruvate concentration on

the basis of the experimental standard curve. The assay described above was performed at room temperature.

Cofactor Regeneration Using W3110 Δ DA (pTrc-gpsA)

To check the ability of the cofactor regeneration strain (permeabilized cells of W3110 Δ DA (pTrc-gpsA)), the reaction of lactate dehydrogenase was conducted at room temperature with the following conditions: 10% (v/v) glycerol, 10 mM pyruvate, 60 units of lactate dehydrogenase, 50 mM potassium phosphate buffer (pH 8.8), 10% (v/v) permeabilized W3110 Δ DA (pTrc-gpsA) cells, and 1 mM NAD⁺. After 1 h reaction, the DNP assay was conducted to determine the remaining pyruvate concentration. All tests were performed with two sets of duplicate experimental setups.

Statistical Analysis

Comparisons of the means for the assay of G3PDH activity and for the cofactor regeneration using W3110 Δ DA (pTrc-gpsA) were performed using the Student's *t*-test.

Results and Discussion

The overall schematic strategy for development of the cofactor regeneration biocatalyst is summarized in Fig. 1. To generate NADH or NADPH from the glycerol source, the following manipulations were performed. First, the *gldA* gene, which encodes glycerol dehydrogenase, was disrupted to block the conversion of glycerol to dihydroxyacetone and to avoid an outward flow of glycerol. Second, the *glpD* gene, which encodes flavin adenine dinucleotide (FAD)-dependent G3PDH that converts G3P to DHAP with a quinone, ubiquinone, or FAD, was disrupted. Next, the *gpsA* gene, which encodes an NAD(P)⁺-dependent G3PDH that produces DHAP and NAD(P)H from G3P and NAD(P)⁺, was overexpressed using the pTrc-gpsA vector. This strategy was designed to substitute the reaction of the enzyme encoded by *glpD* with the NAD(P)⁺-dependent G3PDH reaction. By switching a reaction utilizing quinone, ubiquinone, or FAD to a reaction

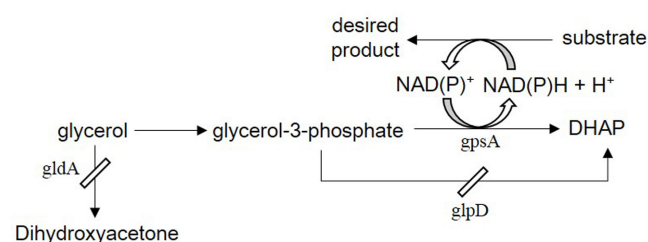


Fig. 1. Overall schematic strategy for development of the cofactor regeneration biocatalyst.

Table 2. NAD(P)H generation by NAD(P)⁺-dependent G3PDH.

			Concentration (μM)		
			<i>t</i> = 0 (-) ^c	<i>t</i> = 0 (+) ^d	<i>t</i> = 30 min
NADH	pH 7	G3PDH ^a	2.745	5.358	15.807
		Control ^b	2.745	4.052	3.678
	pH 9	G3PDH	4.052	13.382	63.764 [#]
		Control	5.358	5.544	5.358
NADPH	pH 7	G3PDH	5.025	9.450	35.794 [*]
		Control	4.824	6.031	6.232
	pH 9	G3PDH	5.226	22.119	70.785 [#]
		Control	5.226	6.433	8.645

Table values represent the mean of data obtained from two sets of duplicate experimental setups. **p* < 0.05 (comparison between *t* = 0 (-) and *t* = 30 min), #*p* < 0.05 (comparison between pH 7 and pH 9).

^aG3PDH activity was determined using Tuner (pCDF-gpsA) cell lysates.

^bControl activity was determined using Tuner cell lysates containing only the pCDFDuet vector.

^cBefore the addition of cell lysates.

^dImmediately after the addition of cell lysates.

employing NAD(P)⁺ as a cosubstrate, the regeneration of the desired cofactor, NAD(P)H, could be obtained from the glycerol source.

Measurement of G3PDH Activity for NAD(P)H Generation

The G3PDH activity for NAD(P)H generation from G3P and NAD(P)⁺ was measured at room temperature in vitro (Table 2). The most noteworthy is that G3PDH was able to generate both NADH and NADPH. With cell lysates obtained from cells carrying the control plasmid, NAD(P)H was not generated in all conditions. On the other hand, with cell lysates obtained from cells carrying the pCDF-gpsA plasmid, significant changes in NAD(P)H concentrations were detected. To compare the effect of pH, the G3PDH activity was examined in pH 7 and 9 conditions. As shown in Table 2, NADH generation was approximately 4 times

higher and NADPH generation was 2 times higher at pH 9 than at pH 7. Therefore, with an abundant supply of G3P, both NADH and NADPH generation could be achieved by G3PDH, and more effectively at pH 9.

Coupling Reaction for Evaluation of Cofactor Regeneration Efficiency

The lactate dehydrogenase reaction was selected as a coupling reaction to evaluate the efficiency of cofactor regeneration. Lactate dehydrogenase consumes NADH to produce lactate from pyruvate, for which the concentration can be easily assayed by a colorimetric method [19]. The generation of NADH by G3PDH enables the conversion of pyruvate to lactate via the consumption of NADH. Thus, a decrease in pyruvate concentration indicates the efficiency of cofactor regeneration. To demonstrate the relationship between pyruvate and NADH consumption, we tested whether pyruvate consumption was proportional to the consumption of NADH. As shown in Table 3, the consumption of NADH was proportional to the consumption of pyruvate and the coefficient of determination (*R*² value) between them was 0.9988. These results indicated that the degree of NADH regeneration by the G3PDH reaction could be estimated by pyruvate consumption, which is easily determined through the DNP assay.

NADH Regeneration with Permeabilized W3110ΔDA (pTrc-gpsA)

The ability of permeabilized W3110ΔDA (pTrc-gpsA) to regenerate NADH was evaluated by determining the conversion of pyruvate by the lactate dehydrogenase reaction. After adding glycerol, NAD⁺ and pyruvate were added, and W3110ΔDA (pTrc-gpsA) or lactate dehydrogenase was introduced to the reaction mixture at room temperature. As shown in the (B) condition of Fig. 2, lactate dehydrogenase rarely utilized pyruvate in the absence of the cofactor regeneration biocatalyst, W3110ΔDA (pTrc-gpsA). On the

Table 3. Linear correlation between the consumptions of pyruvate and NADH in the lactate dehydrogenase reaction.

Input NADH	Residual pyruvate	Pyruvate consumption	Residual NADH	NADH consumption
(mM)	Abs. (445 nm)	(mM)	Abs. (340 nm)	(mM)
0	0.546	0	0.168	0
1	0.515	0.55	0.288	0.95
3	0.442	1.86	0.548	2.90

Table values were obtained from two sets of duplicate experimental setups.

The 1 ml reaction mixture used for the assay contained 50 mM potassium phosphate buffer (pH 7.0), 0–3 mM NADH, 10 mM pyruvate, and 25 units of lactate dehydrogenase enzyme. Absorbance (Abs.) was measured after 1 h of reaction at 25°C. Abs. values were converted to residual concentrations on the basis of standard curves and then consumption concentrations were calculated by subtracting from the initial concentration.

The coefficient of determination (*R*² value) between the consumptions of pyruvate and NADH was 0.9988.

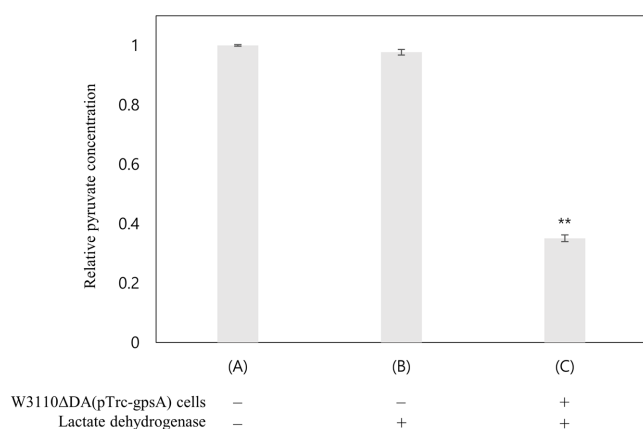


Fig. 2. NADH regeneration through the G3PDH reaction of W3110ΔDA (pTrc-gpsA).

The reaction conditions were as follows: 10% (v/v) glycerol, 10 mM pyruvate, 60 units of lactate dehydrogenase, 50 mM potassium phosphate buffer (pH 8.8), 10% (v/v) permeabilized W3110ΔDA (pTrc-gpsA) cells, and 1 mM NAD⁺. All tests were performed with two sets of duplicate experimental setups at room temperature. The bars indicate the means and SEM. ***p* < 0.01.

other hand, a significant decrease in pyruvate concentration was detected in the (C) condition, where NADH was regenerated through the G3PDH reaction of W3110ΔDA (pTrc-gpsA) and then lactate dehydrogenase consumed NADH to convert pyruvate to lactate. This result indicates that the permeabilized W3110ΔDA (pTrc-gpsA) cells can be used successfully as the cofactor regeneration biocatalyst using cost-competitive glycerol.

The cofactor regeneration system in this study has several powerful advantages over traditional approaches. First, the use of glycerol as a reducing agent is more cost-effective than the use of other sacrificial substrates, such as formic acid, glucose, and sodium phosphite. Second, as shown in Table 2, NADPH regeneration is also attainable by NAD(P)⁺-dependent G3PDH, which is overexpressed in the whole-cell catalyst developed in this study. In future, if experimental evolution of G3PDH is necessary to improve the cofactor regeneration efficiency, the pyruvate colorimetric assay would provide a simple screening method for efficient enzymes, without requiring the development of another screening system. Lastly, the permeabilization of the cofactor regeneration biocatalyst provides a more stable environment for the desired reaction while overcoming the cell membrane barrier.

In this study, a novel whole-cell catalyst for cofactor regeneration was developed, and its ability to regenerate NAD(P)H was verified. To utilize economical glycerol as a

sacrificial substrate, the *gpsA* gene, encoding NAD(P)⁺-dependent G3PDH, was overexpressed in a strain containing deletions of the *glpD* and *gldA* genes. These manipulations were designed to block the outflow of the glycerol source and to substitute the NAD(P)H-generating reaction of G3PDH for reactions of the enzyme encoded by *glpD*. The cofactor regeneration efficiency was evaluated using a simple colorimetric assay to detect changes in the pyruvate concentration. NADH produced by the NAD(P)⁺-dependent G3PDH was consumed by lactate dehydrogenase, and then NAD⁺ was regenerated to NADH by NAD(P)⁺-dependent G3PDH, as designed. In conclusion, we have demonstrated the feasibility of NAD(P)⁺-dependent G3PDH-catalyzed cofactor regeneration in permeabilized *E. coli* cells. The developed cofactor regeneration biocatalyst is applicable to a wide variety of oxidoreductase reactions that require NAD(P)H.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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