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Production of dihydroxyacetone from glycerol by engineered *Escherichia coli* cells co-expressing *gldA* and *nox* genes

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Glycerol can be converted into more valuable compound dihydroxyacetone by the nicotinamide adenine dinucleotide (NAD^+)-dependent glycerol dehydrogenase. However, it is economically prohibitive to produce dihydroxyacetone using purified glycerol dehydrogenase at the expense of a stoichiometric amount of the cofactor NAD^+. In this study, *Escherichia coli* was engineered for dihydroxyacetone production by enhancing its glycerol dehydrogenase activity and introducing NADH oxidase activity. Under optimized conditions, dihydroxyacetone productivity reached 0.13 g/h/g wet cell mass by recombinant *E. coli* D4 (pET-24b-gldA+nox) cells co-expressing *gldA* gene from *E. coli* and *nox* gene from *Enterococcus faecalis*. It was interesting to note that exogenous NAD^+ greatly improved dihydroxyacetone production for the whole-cell biotransformation process. These results should be useful for the development of advanced bioprocess in terms of glycerol utilization.

Key words: Dihydroxyacetone, Glycerol dehydrogenase, NAD^+, whole-cell biotransformation, *Escherichia coli*.

INTRODUCTION

As glycerol is a relatively cheap and readily available commodity from the biodiesel industry, it is attractive to convert glycerol into value-added products, such as 1,3-propanediol (Willke and Vorlop, 2008), glyceric acid (Habe et al., 2009a), xylulose (Habe et al., 2009b) and dihydroxyacetone (DHA) (Li et al., 2010). DHA is a versatile product widely used in cosmetic, pharmaceutical and chemical industries. For example, superficial skin tanning generated by application of DHA may delay skin cancer development. Based on its multiple function and expensive price, DHA production has a great developmental potential and a large market space. Biological

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Abbreviations: DHA, Dihydroxyacetone; **NAD^+,** nicotinamide adenine dinucleotide; **GDH,** glycerol dehydrogenase; **NOX,** NADH oxidase; **IPTG,** isopropyl-β-D-thiogalactoside; **LB,** Luria-Bertani; **Kan,** kanamycin; **WCM,** wet cell mass; **SDS-PAGE,** sodium dodecyl sulfate/polyacrylamide gel electrophoresis.

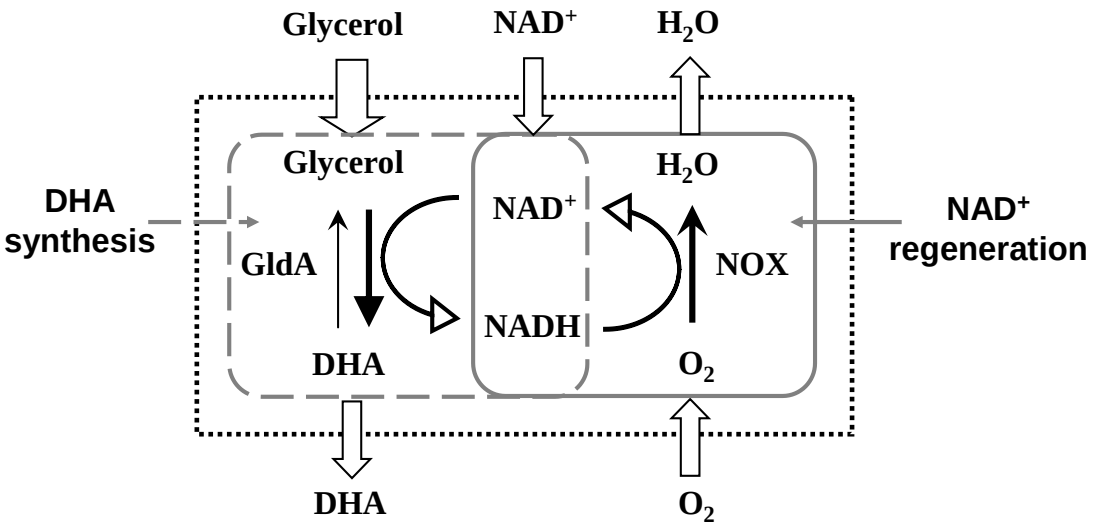


Figure 1. Schematic diagram of whole-cell biocatalytic oxidation of glycerol to DHA coupled with cofactor regeneration system. The outer dotted rectangle represents the *E. coli* cell membrane with permeability. GDH, Glycerol dehydrogenase (EC 1.1.1.6) from *E. coli*; NOX, NADH oxidase (EC 1.6.3.1) from *E. faecalis*.

production of DHA from glycerol can be realized by the action of nicotinamide adenine dinucleotide (NAD^+)-dependent glycerol dehydrogenase (GDH) (EC 1.1.1.6) or pyrroloquinoline quinone (PQQ)-dependent GDH (EC 1.1.99.22) (Claret, 1994; Subedi et al., 2008). There are a number of studies on the production of DHA using *Gluconobacter oxydans* as the host because it produces native PQQ-dependent GDH (Gätgens et al., 2007; Hu et al., 2010; Li et al., 2010).

However, the production of DHA using engineered *Escherichia coli* cells, with clear genetic background and mature genetic manipulation platform, has not been explored. Based on the fact that it is a widely used strain in various biotechnological processes, and can be in high cell density fermentation, it seemed worthwhile testing the possibility for the conversion of glycerol into DHA by *E. coli*.

The NAD^+-dependent GDH, encoded by the *gldA* gene (Accession No. CP000948) in *E. coli*, catalyzes reversible reactions for the interconversion of glycerol and DHA. In the forward reaction, glycerol is oxidized to DHA at the expense of a stoichiometric amount of the cofactor NAD^+. Therefore, NAD^+ supply and recycling is essential to drive the forward reaction for DHA accumulation. NADH oxidase (NOX) is known to catalyze the oxidation of NADH to NAD^+ using oxygen as the terminal oxidant. In the literature, NOX has been used to couple other NADH-producing enzymes for cofactor recycling (Xiao et al., 2010). In *Enterococcus faecalis*, NOX is encoded by the *nox* gene (Accession No. CP002491), and the products of NOX are NAD^+ and H_2O (Ross and Claiborne, 1992).

Due to the protection from the external environment and convenient operation, whole-cell catalysts are more attractive than purified enzymes or crude cell extracts. In this study, results on engineering of *E. coli* for DHA production by enhancing its GDH activity and introducing NOX activity were reported. In this system, NAD^+ was spontaneously regenerated during DHA production by the action of NOX using air as the terminal oxidant (Figure 1). Then, the effects of initial pH and exogenous NAD^+ on DHA production were investigated.

MATERIALS AND METHODS

Materials, strains and culture conditions

Oligonucleotides used in this study (Table 1), restriction endonucleases, DNA polymerase and isopropyl-β-D-thiogalactoside (IPTG) were purchased from TaKaRa (Dalian, China). Sequence analysis was performed by Sangon (Shanghai, China) or TaKaRa. The kits for DNA purification, gel recovery or plasmid mini-preparation were obtained from Beyotime (Jiangsu, China). DHA standard (1,3-dihydroxyacetone dimer) was obtained from Sigma (St. Louis, MO, USA). NAD^+ and NADH standards were purchased from DingGuo (Beijing, China). Other reagents were purchased from Bonuo (Dalian, China).

E. coli BL21 (DE3) cells were transformed by plasmids pET-24b, pET-24b-nox, pET-24b-gldA and pET-24b-gldA+nox to give recombinant strains D1 (control strain), D2 (*E. coli* expressing NOX), D3 (*E. coli* expressing GDH) and D4 (*E. coli* expressing GDH and NOX) (Table 2). *E. coli* BL21(DE3) cells were used throughout for cloning and expression. *E. coli* strains were cultured in Luria-Bertani (LB) broth at 37°C unless stated otherwise. Kanamycin (Kan, 50 μg/ml) was applied for the selection of recombinant strains.

Plasmid construction

The *gldA* gene was amplified from *E. coli* genomic DNA using primers gldA-F-Nde I/ gldA-R-Xho I; the *nox* gene was amplified

Table 1. Primers used in this study.

Primer name	Sequence (5'-3') [a]
nox-F-Nde I	TAC CAT ATG AAA GTC GTA GTC GTA GGA
nox-R-BamH I	ATA GGA TCC TTA GTG GTG GTG GTG GTG GTG CAT ATT TTC TAA AGC GGC T
gldA-F-Nde I	AGC ACA TAT GGA CCG CAT TAT TC
gldA-R-Xho I	CCG CTC GAG TTC CCA CTC TTG CAG
gldA-R-rbs	GTG TAT ATC TCC TTG AAT TCG GAT CC TTA TTC CCA CTC TTG CAG
nox-F-rbs	GGA TCC GAA TTC AAG GAG ATA TAC AC ATG AAA GTC GTA GTC GTA GGA
nox-R-Xho I	CCG CTC GAG TTA CAT ATT TTC TAA AGC GGC T
T7	TAA TAC GAC TCA CTA TAG G
T7ter	GCT AGT TAT TGC TCA GCG G

[a]Primers containing the restriction sites are shown by: Nde I (single underline), Xho I (double underline) and BamH I (dotted underline) were underlined.

Table 2. Strains and plasmids used in this study.

Strain/plasmid	Description[a]	Source
Strain		
E. coli BL21(DE3)	*F⁻ ompT hsdS$_B$ ($r_B^-m_B^-$) gal dcm* (DE3), expression host	Novagen
D1	BL21(DE3) containing pET-24b, control strain	This study
D2	BL21(DE3) containing pET-24b-nox, NOX expression strain	This study
D3	BL21(DE3) containing pET-24b-gldA, GDH expression strain	This study
D4	BL21(DE3) containing pET-24b-gldA+nox, GDH and NOX co-expressed strain	This study
Plasmids		
pET-24b	Expression vector/T7 promoter, Kan^r	Novagen
pET-24b-nox	pET-24b carrying *nox* gene	This study
pET-24b-gldA	pET-24b carrying *gldA* gene	This study
pET-24b-gldA+nox	pET-24b carrying *gldA* and *nox* genes	This study
pET-24b-nox-fdh	pET-24b carrying *nox* and *fdh* genes	Lab collection

[a]Kan^r, Kanamycin resistance, *gldA* gene was obtained from *E. coli*, *nox* gene was obtained from *E. faecalis*.

from pET-24b-nox-fdh using primers nox-F-Nde I/nox-R-BamH I. The polymerase chain reaction (PCR) products were purified, digested with Nde I/Xho I and Nde I/ BamH I, and cloned into plasmid pET-24b digested with the same enzymes to give pET-24b-gldA and pET-24b-nox, respectively (Table 2). pET24b-gldA+nox was constructed by double-joint PCR method with minor modifications (Yu et al., 2004). For the first round PCR, *gldA* gene and *nox* gene were subcloned from pET-24b-gldA and pET-24b-nox using primers gldA-F-Nde I/gldA-R-rbs and nox-F-rbs/nox-R-Xho I, respectively. For the second round PCR, *gldA* and *nox* genes from the first round PCR were fused by overlapping sequence within the 5' ends of primers gldA-R-rbs and nox-F-rbs. For the last round PCR, products of the second round PCR were used as template to amplify fused genes using primers gldA-F-Nde I/nox-R-Xho I. The fused genes were digested with Nde I/Xho I, and cloned into plasmid pET-24b digested with the same enzymes. Two genes were verified by sequence analysis using primers T7 and T7ter.

Preparation of resting cells

Recombinant strains were cultured in LB medium supplemented with 50 μg/ml Kan. When the optical density at 600 nm reached 0.6 to 0.8, IPTG was added to a final concentration of 0.5 mM to induce gene expression. Then the strains were incubated at 30°C for 20 h. Cells were harvested by centrifugation at 17,800 *g* for 5 min, washed twice with sterilized water and resuspended in 100 mM phosphate buffer (pH 8.0), stored at 4°C and employed as the whole-cell biocatalysts within 24 h.

DHA production

The biotransformation reactions were carried out in test tubes with shaking (200 rpm) at 37°C. The reaction mixtures contained 10 g wet cell mass (WCM)/l, 10 g/L glycerol and 5.0 mM NAD^+ in 100 mM phosphate buffer (pH 8.0). To test the effect of the initial pH on DHA production, reactions were carried out in phosphate buffer (K_2HPO_4-KH_2PO_4) with pH ranging from 6.0 to 9.0. To test the effect of exogenous NAD^+ on DHA production, the reactions were carried out in the presence of different amounts of exogenous NAD^+ ranging from 0.1 to 10.0 mM in 100 mM phosphate buffer (pH 9.0). To test the intracellular NAD^+ concentration, the reaction mixture contained 10 g WCM/l, 20 g/L glycerol and 5.0 mM NAD^+ in 100 mM phosphate buffer (pH 9.0).

Analytic methods

All recombinant strains were checked for the expression of targeted

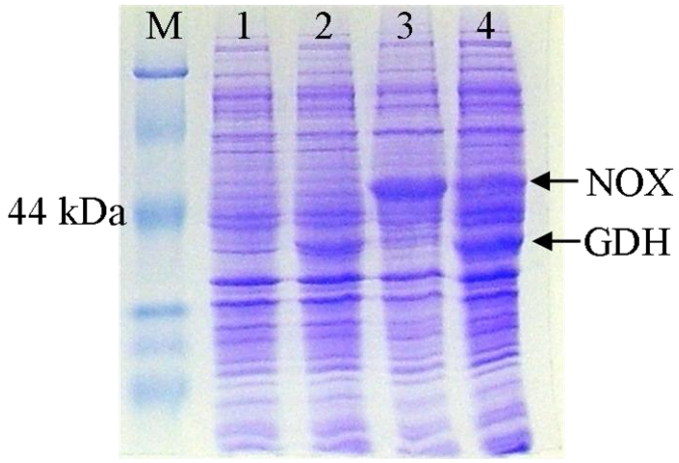


Figure 2. SDS-PAGE analysis of recombinant *E. coli* BL21(DE3) strains. Lane M, Molecular weight marker 14 to 97 kDa; lane 1, D1 (control strain); lane 2, D3 (*E. coli* expressing GDH); lane 3, D2 (*E. coli* expressing NOX); lane 4, D4 (*E. coli* expressing GDH and NOX).

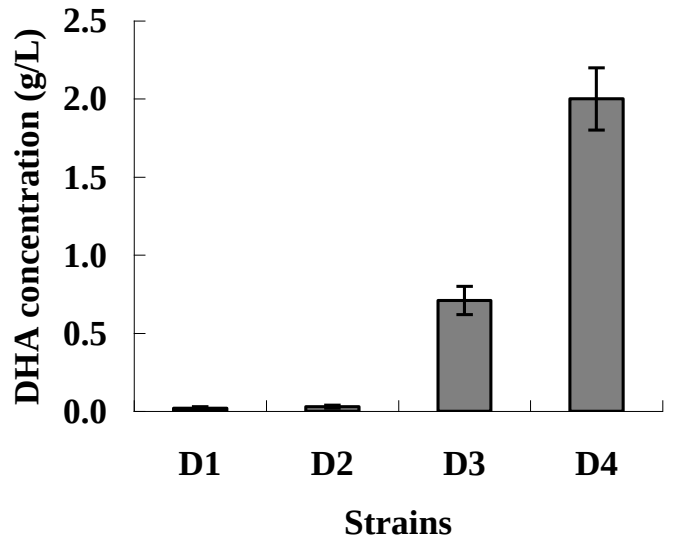


Figure 3. DHA production of the whole-cell biocatalyste. D1, Control strain; D2, *E. coli* expressing NOX; D3, *E. coli* expressing GDH; D4, *E. coli* expressing GDH and NOX. Error bars depict standard deviation of three duplicate trials.

proteins by analyzing their corresponding crude cell extracts sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). GDH or NOX activity in the crude cell extract was measured with a Jasco V-530 spectrophotometer by monitoring the absorbance changes at 340 nm over time in 100 mM Tris-HCl (pH 8.0) (Hirano et al., 2008; Subedi et al., 2008). For GDH activity assay, reactions were performed in the presence of 10 mM glycerol and 1.0 mM NAD^+, and for NOX, in the presence of 0.1 mM NADH.

DHA concentration was determined by the absorbance at 620 nm (A_{620}) using a visible spectrophotometric method with minor modifications (Liu et al., 2008). Briefly, samples of the biotransformation mixtures were centrifuged at 16,100 *g* for 1 min. The supernatant samples in 20 µl each were mixed with 180 µl assay solution which contain 1% (w/v) diphenylamine and 10% (v/v) sulfuric acid in acetic acid, followed by heating at 105°C for 20 min, and then cooled to room temperature. The A_{620} values were recorded for each sample, and DHA concentrations were quantified according to a standard curve obtained under identical conditions. Intracellular concentrations of NAD(H) were estimated by using the cofactor recycling assay according to a published procedure (Zhou et al., 2011).

RESULTS AND DISCUSSION

Construction of the whole-cell biocatalyst

The genes encoding GDH and NOX were cloned and co-expressed in *E. coli* BL21(DE3) to give the designated strain D4. In order to compare the performance between D4 and the strains with enhanced GDH or NOX activity only, *gldA* or *nox* gene was expressed in Bl21(DE3) to form strain D3 and D2, respectively. *E. coli* BL21(DE3) containing the cloning plasmid pET-24b without a DNA insert was designated as D1 for a negative control (Table 2). SDS-PAGE analysis indicated that strains D2, D3 and D4 overproduced the targeted protein upon IPTG induction (Figure 2). Two protein bands were evident with a molecular size of approximately 39 and 50 kDa, which were in well agreement with expected molecular weight for GDH and NOX, respectively. In addition, the activities of GDH or NOX in crude cell extracts were observed (data not shown), indicating that both proteins were overexpressed in active forms. Therefore, those recombinant strains were competent to be explored as whole-cell catalysts.

Production of DHA from glycerol

To investigate DHA production by the constructed biocatalysts, reactions were performed using the resting cells from strains D1, D2, D3 and D4, and results are shown in Figure 3. For strains D1 and D2, DHA concentrations were barely above the background, that is 0.02 ± 0.01 g/L for strain D1 and 0.03 ± 0.01 g/L for strain D2. These data suggests that native GDH in *E. coli* BL21(DE3) was extremely low, such that the presence of excess NOX activity failed to drive the oxidation of glycerol to DHA. However, the strain D3 harboring a plasmid borne *gldA* gene produced 0.71 ± 0.09 g/L DHA, indicating that a higher GDH protein level was key to turnover glycerol. In the case of the strain D4, DHA concentration was 1.99 ± 0.20 g/L, about 2.8-fold higher than that of the strain D3. This result indicates that the presence of NOX substantially facilitated the oxidation of glycerol by GDH.

pH effect on DHA biosynthesis

The initial pH value of the reaction buffer had significant

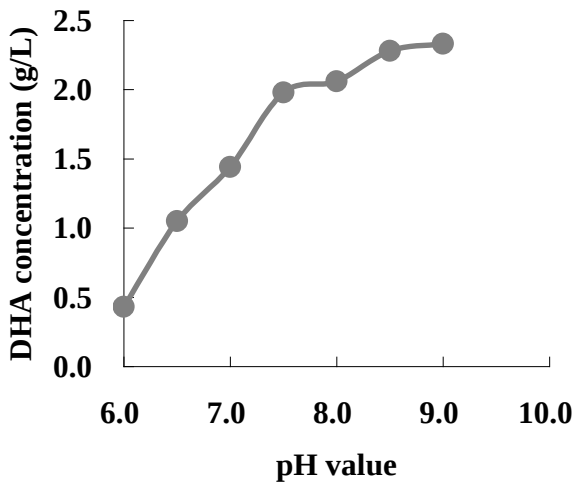
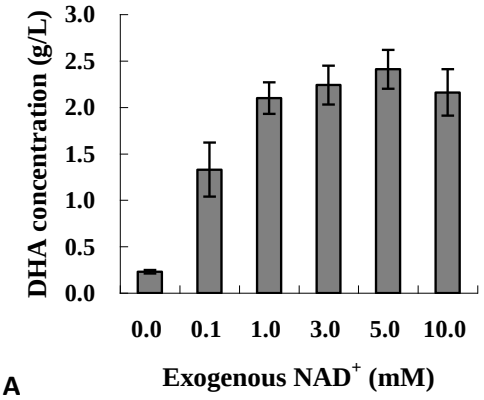
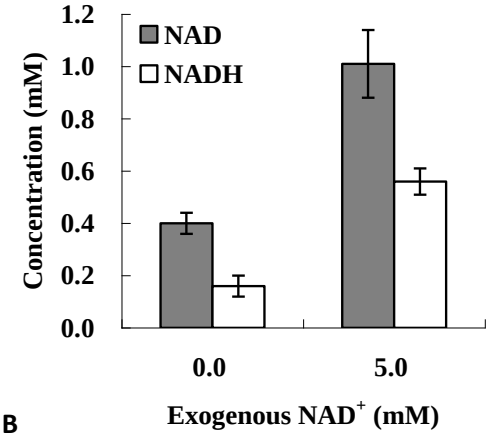


Figure 4. Effects of initial pH on DHA production. Initial pH of reaction buffer was from 6.0 to 9.0. Each point was the average value of two independent experiments.



A



B

Figure 5. Exogenous NAD^+ effect on DHA production. **A.** DHA production with initial NAD^+ concentration from 0.1 to 10.0 mM. **B.** intracelllar NAD(H) concentration with or without exogenous 5.0 mM NAD^+. Error bars depict standard deviation of three duplicate trials.

affects on DHA production. Early study showed that the optimal pH for the oxidation of glycerol by purified native GDH was in the range of 9.5 to 10.0, with a rapid decline of the activity below pH 8.5 (Tang et al., 1979). DHA production with the strain D4 in pH range of 6.0 to 9.0 were evaluated (Figure 4). It was clear that DHA production reached a plateau at pH 8.5 to 9.0, suggesting that the optimum pH for the whole-cell biocatalysis system was lower than the enzymatic system.

Effect of exogenous NAD^+ on DHA biosynthesis

DHA production experiments were also performed in the absence and presence of different amounts of exogenous NAD^+ using the strain D4 as the whole-cell biocatalyst, and results were shown in Figure 5. When no NAD^+ was supplemented in the reaction buffer, DHA concentration was 0.23 ± 0.02 g/L (Figure 5A).

The presence of exogenous NAD^+ significantly improved DHA production. Even the initial NAD^+ concentration in the reaction buffer was only 0.1 mM, DHA concentration increased by 5.7-fold to 1.33 ± 0.23 g/L. The application of higher exogenous NAD^+ concentration further improved DHA production, and the highest DHA concentration was 2.41 ± 0.21 g/L when initial NAD^+ concentration was 5.0 mM. However, when NAD^+ concentration reached 10.0 mM, DHA concentration dropped to 2.16 ± 0.25 g/L.

Cell samples were collected at the end of the reaction and deter-mined intracellular NAD(H) concentrations and results are shown in Figure 5B. In the case of no NAD^+ supplementation, NAD^+ and NADH concentrations were 0.40 ± 0.04 and 0.16 ± 0.04 mM, respectively, with an NAD^+/NADH ratio of 2.5. When 5.0 mM NAD^+ was added in the buffer, intracellular NAD^+ and NADH concentrations reached 1.01 ± 0.13 and 0.56 ± 0.05 mM, respectively, with an NAD^+/NADH ratio of 1.8.

The fact that in both cases the NAD^+/NADH ratio were higher than one suggested that the intracellular environmental remained oxidative. Therefore, the cessation of DHA production was not likely due to the limitation of NAD^+ supply. It was clear that total intracellular cofactor concentration was doubled in the case with NAD^+ supplementation compared with that of the sample without NAD^+ supplementation, indicating that exogenous NAD^+ was uptaken by *E. coli* cells under the reaction conditions.

It is generally believed that NAD^+ cannot be uptaken by microorganisms. To enable *E. coli* cells to utilize exogenous NAD^+, one may introduce NAD^+-specific transporter (Zhou et al., 2011), or alternatively, permea-bilize cells by treating with polymyxin B sulfate (Kratzer et al., 2008) or ethylene diamine tricholoroacetic acid (EDTA)-toluene mixture (Zhang et al., 2009). It is interes-ting to understand the mechanism of how did exogenous NAD^+ get into the cell and improve DHA production in the absence of these conventional permeating agents.

Conclusions

In this work, *E. coli* strains were engineered for the aerobic oxidation of glycerol to DHA using a whole-cell biotransformation process. Under optimized conditions, DHA productivity reached 0.13 g/h/g wet cell mass. It was shown that the presence of extracellular NAD^+ significantly improved DHA production. These results provide valuable information for the development of robust strains for glycerol utilization.

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