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RESEARCH ARTICLE

Research of 1,3-Dihydroxyacetone Production by Overexpressing Glycerol Transporter and Glycerol Dehydrogenase

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Abstract

1,3-Dihydroxyacetone (DHA), a natural ketose, is widely used in the chemical, cosmetic, and pharmaceutical industries. The current method for DHA production is *Gluconobacter oxydans* (*G. oxydans*) fermentation, but the high concentration of glycerol in the fermentation broth inhibits cells growth. To overcome this obstacle, in this study, we overexpressed the glycerol transporter (GlpFp) by the use of promoters P_{tufB}, P_{gmr}, P_{glp1}, and P_{glp2} in *G. oxydans* 621H. The results show that the glycerol tolerances of strains overexpressing *GlpF* were all much better than that of the control strain. The glycerol dehydrogenase gene (*Gdh*) was overexpressed by the promoters P_{tufB} and P_{gdh}, which increased the DHA titer by 12.7% compared with that of the control group. When *GlpF* and *Gdh* genes were co-overexpressed in *G. oxydans* 621H, the OD600 value of the engineered strains all increased, but the DHA titers decreased in different degrees, as compared with strains that overexpressed only *Gdh*. This study provides a reference for future research on DHA production.

Keywords *G. oxydans* 621H · 1,3-dihydroxyacetone · Glycerol · Glycerol transporter · Glycerol dehydrogenase

Introduction

1,3-Dihydroxyacetone (DHA), the simplest ketose with two hydroxyl groups and one ketone group, participates in many kinds of chemical reactions due to its active chemical properties. DHA is reported to be widely used in the cosmetics industry for its skin protection ability [1–3]. Also, DHA can improve human endurance [4–6], detoxify poisonous substances [7, 8], and fight viruses [9], so it is also widely used in the pharmaceutical [10] and chemical industries [11]. DHA can also be used as a precursor for synthesizing various chemicals such as methotrexate, lactic acid, surfactants, 1,2-propylene glycol, and pharmaceutical products [12].

Currently, microbial fermentation is the most common method used to produce DHA. *G. oxydans* is widely used for its high DHA production via the incomplete oxidation of glycerol. For instance, Tanamool et al. [13] isolated a *G. oxydans* NKC115 strain to produce DHA for which the DHA titer reached 27.50 g/L with an initial crude glycerol concentration of 100 g/L. However, the high glycerol substrate concentration inhibited the cell growth rate and the conversion efficiency of glycerol [14–17]. A high concentration of DHA affects the uptake rate of glycerol, thereby affecting the growth and metabolism of cells and indirectly reducing the DHA titer [18, 19]. For example, increasing the initial glycerol concentration from 31 to 129 g/L was reported to decrease the specific growth rate of cells by 70% and decrease the maximum specific production rate of DHA by 30% [15]. To prevent the glycerol inhibition effect, Dikshit et al. [20] used immobilized *G. oxydans* cells to produce DHA by batch and repeated batch fermentation and obtained a final DHA titer of 17.83 g/L, which was almost ninefold higher than that of the control groups. Researchers have also attempted to improve the metabolic pathways of DHA-producing strains by overexpressing or inhibiting-related genes [21, 22]. For instance, the *G. oxydans* membrane dehydrogenase gene *sldAB* was overexpressed using the promoters P_{gdh} and P_{tufB}, respectively. When 550 mmol/L glycerol was

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used as the substrate, the accumulation of DHA in the fermentation broth increased by at least 25% compared with the control group [21].

The glycerol transporter and glycerol dehydrogenase are two important enzymes in DHA synthesis. The glycerol transporter encoded by *GlpF* is an aquaporin, which makes the uptake of glycerol more efficient [23–25]. The glycerol transporter is very sensitive to DHA [26]. Therefore, the inhibition of DHA on the glycerol transporter is the most important factor for inhibiting *G. oxydans* growth and is thus also the main inhibition effect in DHA production [27]. Overexpressing the glycerol transporter in *Escherichia coli* (*E. coli*) effectively increases the permeability of glycerol and promotes cell growth [28]. Glycerol dehydrogenase encoded by *Gdh* is a key enzyme for DHA synthesis in *G. oxydans*. This enzyme consists of two subunits and employs oxygen as the final acceptor of reduced equivalents without NADH mediation. Glycerol can be directly oxidized into DHA by the glycerol dehydrogenase present in the cell membrane. Wei et al. [29] amplified *Gdh* and *Ndh*, constructed the plasmids pET-Gdh and pET-Ndh, and transfected them into *E. coli* to obtain the highest DHA titer of 85 g/L. In view of the importance of the glycerol transporter and glycerol dehydrogenase in synthesizing DHA, in this study, we overexpressed *GlpF* and *Gdh* in *G. oxydans* via promoter engineering. The promoter P_{tufB} from *E. coli* was reported to successfully enhance the production of 2-keto-L-gulonate in *G. oxydans* [30], and P_{gmr} from plasmid pBBR1MCS-5 is another frequently used promoter in *G. oxydans*. First, we overexpressed *GlpF* by changing the promoters P_{tufB}, P_{gmr}, P_{glp1}, and P_{glp2} to promote the growth of *G. oxydans* and improve the glycerol tolerance of *G. oxydans*. Next, we overexpressed *Gdh* using promoters P_{tufB} and P_{gdh} to enhance the ability of *G. oxydans* in DHA production. Lastly, we co-expressed *GlpF* and *Gdh* in *G. oxydans* and found all the resulting strains to have a higher OD600 value than strains only expressing *Gdh*.

Materials and Methods

Strains, Medium, and Culture Conditions

We used *G. oxydans* 621H (wild-type strain) as the original strain for DHA production and *E. coli* DH5a for plasmid construction. We used *E. coli* HB101 as the assistant bacteria for transformation. All of these strains were preserved in our laboratory. Table 1 lists all of the engineered strains used in this work.

To cultivate strains of *E. coli* DH5a and *E. coli* HB101, we used an LB medium containing 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl.

We used a sorbitol medium containing 5 g/L sorbitol, 20 g/L yeast extract, 5 g/L $(NH_4)_2SO$ [1, 4], 0.5 g/L KH_2PO_4, and 0.5 g/L $MgSO_4{\cdot}7H_2O$ to cultivate *G. oxydans* 621H and select engineered strains by adding appropriate concentrations of antibiotics.

We used a fermentation medium containing 100 g/L glycerol, 2.5 g/L yeast extract, 2 g/L peptone, 2 g/L $(NH_4)_2SO_4$, 2.62 g/L $KH_2PO_4{\cdot}3H_2O$, 1 g/L $MgSO_4{\cdot}7H_2O$, 0.56 g/L $MnSO_4{\cdot}H_2O$, 2.5 g/L $CaCO_3$, and 1.25 g/L $CaCl_2$ to cultivate the engineered strains for 48 h at 30 °C, 220 r/min.

Table 1 Strains used in this work

Strain	Relevant characteristics	Source/References
E. coli DH5a	supE44 ΔlacU169(ø80lacZΔM15) hsdR17 recA1 endA1gyrA96 thi-I relA1	[31]
E. coli HB101	supE44 hsdS20($r_B^-m_B^-$)recA13 ara-14 proA2 lacY1 galK2rpsL20 xyl-5 mtl-1	[32]
G. oxydans 621H	Wild-type strain	[33]
G1	Plasmid pBBR-P_{tufB}-GlpF was delivered into *G. oxydans* 621H	This study
G2	Plasmid pBBR-P_{gmr}-GlpF was delivered into *G. oxydans* 621H	This study
G3	Plasmid pBBR-P_{glp1}-GlpF was delivered into *G. oxydans* 621H	This study
G4	Plasmid pBBR-P_{glp2}-GlpF was delivered into *G. oxydans* 621H	This study
G5	Plasmid pBBR-P_{gdh}-Gdh was delivered into *G. oxydans* 621H	This study
G6	Plasmid pBBR-P_{tufB}-Gdh was delivered into *G. oxydans* 621H	This study
G7	Plasmid pBBR-P_{tufB}-GlpF-P_{tufB}-Gdh was delivered into *G. oxydans* 621H	This study
G8	Plasmid pBBR-P_{gmr}-GlpF-P_{tufB}-Gdh was delivered into *G. oxydans* 621H	This study
G9	Plasmid pBBR-P_{glp1}-GlpF-P_{tufB}-Gdh was delivered into *G. oxydans* 621H	This study
G10	Plasmid pBBR-P_{glp2}-GlpF-P_{tufB}-Gdh was delivered into *G. oxydans* 621H	This study
G11	Plasmid pBBR-P_{tufB}-GlpF-P_{gdh}-Gdh was delivered into *G. oxydans* 621H	This study
G12	Plasmid pBBR-P_{gmr}-GlpF-P_{gdh}-Gdh was delivered into *G. oxydans* 621H	This study
G13	Plasmid pBBR-P_{glp1}-GlpF-P_{gdh}-Gdh was delivered into *G. oxydans* 621H	This study
G14	Plasmid pBBR-P_{glp2}-GlpF-P_{gdh}-Gdh was delivered into *G. oxydans* 621H	This study

DNA Manipulation

We used the promoters P_{glp1} and P_{glp2} as native *GlpF* promoters and P_{gdh} as the promoter of *Gdh*. The glycerol transporter gene *GlpF*, glycerol dehydrogenase gene *Gdh*, and DNA fragments containing *GlpF1*(P_{glp1}-*GlpF*), *GlpF2*(P_{glp2}-*GlpF*), *Gdh1*(P_{gdh}-*Gdh*), and promoter P_{tufB} were amplified from the genomic DNA of *G. oxydans* 621H. The promoter P_{gmr} was amplified from the plasmid pBBR1MCS-5.

Table 2 shows a summary of all the primers used in this work.

Plasmids and Strains Construction

To obtain the plasmids pBBR-P_{glp1}-GlpF and pBBR-P_{glp2}-GlpF, we inserted DNA fragments of *GlpF1*(P_{glp1}-*GlpF*) and *GlpF2*(P_{glp2}-*GlpF*) into the plasmid pBBR1MCS-5 digested by the restriction endonucleases *Hin*dIII and *Eco*RI, respectively.

The DNA fragments P_{tufB} and P_{gmr} were digested by the restriction endonucleases *Xho*I and *Nde*I, and *GlpF* was digested by the restriction endonucleases *Nde*I and *Eco*RI. We obtained the DNA fragments P_{tufB}-*GlpF* and P_{gmr}-*GlpF* using T4 DNA ligase and inserted them into the plasmid pBBR1MCS-5 digested by the restriction endonucleases *Xho*I and *Eco*RI to obtain plasmids pBBR-P_{tufB}-GlpF and pBBR-P_{gmr}-GlpF, respectively.

To obtain the plasmid pBBR-P_{gdh}-Gdh, we inserted the DNA fragment *Gdh1*(P_{gdh}-*Gdh*) into the plasmid pBBR1MCS-5 digested by restriction endonucleases *Xba*I and *Eco*RI.

The DNA fragment P_{tufB} was digested by the restriction endonucleases *Eco*RI and *Bam*HI, and *Gdh* was digested by the restriction endonucleases *Bam*HI and *Xba*I. We obtained the DNA fragment P_{tufB}-*Gdh* using T4 DNA ligase and then inserted it into the plasmid pBBR1MCS-5 digested by the restriction endonucleases *Eco*RI and *Xba*I to obtain the plasmid pBBR-P_{tufB}-Gdh.

We simultaneously inserted *GlpF* and *Gdh* controlled by different promoters into the plasmid pBBR1MCS-5 to obtain different co-expression vectors.

Table 3 shows a summary of all the plasmids used in this work.

To construct engineered strains, we introduced each of the expression vectors into *G. oxydans* 621H by electrotransformation.

We preserved the *G. oxydans* strain 621H and plasmid pBBR1MCS-5 in our laboratory.

Transcriptional Gene Expression Studies by RT-PCR

We cultured the wild-type and engineered strains in sorbitol medium for 24 h at 30 °C, 220 r/min. Then, we obtained the cells by centrifugation and extracted the total RNA using a TianGen RNA kit. We used the total RNA as a template to obtain cDNA and initiated a reverse transcription polymerase chain reaction (RT-PCR) according to the methods reported by Xue et al. [36]. We used the primers listed in

Table 2 Primers used in this work

Primer	Sequence (5–3)	Restriction site
tuf-F	ACTCGAGCGATGGTAAGAAATCCACTGC	*Xho*I
tuf-R	ATAAGCTTCCATATGCCAAAACCCCGCTCCACC	*Nde*I, *Hin*dIII
gmr-F	ATCTCGAGTAGCTGTTTCCTGTG	*Xho*I
gmr-R	CAAGCTTCCATATGTGCTGCTCCATAAC	*Nde*I, *Hin*dIII
GlpF-F	ACCATATGAGTCAAACATCAACCT	*Nde*I
GlpF-R	TGAATTCTTACAGCGAAGCTTTTTG	*Eco*RI
GlpF1-F	CTCAAGCTTACCGAAATCGCTGCTGTTGTC	*Hin*dIII
GlpF1-R	AAGAATTCCCGTGAAGTGGACTACCTC	*Eco*RI
GlpF2-F	GCCAAGCTTACCGCTACTTCACGAAGACCCT	*Hin*dIII
GlpF2-R	AGAATTCGCCCTCCGACCTCCTCGAT	*Eco*RI
tuf1-F	AGAATTCCGATGGTAAGAAATCCACTGC	*Eco*RI
tuf1-R	ATGGATCCAAAACCCCGCTCCACC	*Bam*HI
Gdh1-F	AGGAATTCTCGACATCGGACAGCAAC	*Eco*RI
Gdh1-R	ACTCTAGACCCCGGAAACGACAAAGG	*Xba*I
Gdh-F	ACGGATCCTCACCAATGCCGAATACTTAT	*Bam*HI
Gdh-R	ATTCTAGATCAGCCCTTGTGAT	*Xba*I
16SrRNA-F	GCGGTTGTTACAGTCAGATG	
16SrRNA-R	GCCTCAGCGTCAGTATCG	
GdhRT-F	CCTGCGTAGCCCTGAAGAAAAC	
GdhRT-R	CGAGCCGATGTCATAGTCCC	

Table 3 Plasmids used in this work

Plasmid	Relevant characteristics	Source/References
pBBR1MCS-5	Gm^R, broad-host-range cloning vector	[34]
pRK2013	Km^R, helper plasmid for triparental mating	[35]
pBBR-P_{tufB}-GlpF	Gm^R, a derivative of pBBR1MCS-5, harboring *GlpF* gene under control of tufB promoter	This study
pBBR-P_{gmr}-GlpF	Gm^R, a derivative of pBBR1MCS-5, harboring *GlpF* gene under control of gmr promoter	This study
pBBR-P_{glp1}-GlpF	Gm^R, a derivative of pBBR1MCS-5, harboring *GlpF* gene under control of glp1 promoter	This study
pBBR-P_{glp2}-GlpF	Gm^R, a derivative of pBBR1MCS-5, harboring *GlpF* gene under control of glp2 promoter	This study
pBBR-P_{gdh}-Gdh	Gm^R, pBBR1MCS -5 derivative containing *Gdh* gene cluster controlled by gdh promoter	This study
pBBR-P_{tufB}-Gdh	Gm^R, pBBR1MCS -5 derivative containing *Gdh* gene cluster controlled by tufB promoter	This study
pBBR-P_{tufB}-GlpF-P_{tufB}-Gdh	Gm^R, a derivative of pBBR1MCS-5, harboring *GlpF* and *Gdh* gene under control of tufB promoter	This study
pBBR-P_{gmr}-GlpF-P_{tufB}-Gdh	Gm^R, a derivative of pBBR1MCS-5, harboring *GlpF* gene under control of gmr promoter and *Gdh* gene under control of tufB promoter	This study
pBBR-P_{glp1}-GlpF-P_{tufB}-Gdh	Gm^R, a derivative of pBBR1MCS-5, harboring *GlpF* gene under control of glp1 promoter and *Gdh* gene under control of tufB promoter	This study
pBBR-P_{glp2}-GlpF-P_{tufB}-Gdh	Gm^R, a derivative of pBBR1MCS-5, harboring *GlpF* gene under control of glp2 promoter and *Gdh* gene under control of tufB promoter	This study
pBBR-P_{tufB}-GlpF-P_{gdh}-Gdh	Gm^R, a derivative of pBBR1MCS-5, harboring *GlpF* gene under control of tufB promoter and *Gdh* gene under control of gdh promoter	This study
pBBR-P_{gmr}-GlpF-P_{gdh}-Gdh	Gm^R, a derivative of pBBR1MCS-5, harboring *GlpF* gene under control of gmr promoter and *Gdh* gene under control of gdh promoter	This study
pBBR-P_{glp1}-GlpF-P_{gdh}-Gdh	Gm^R, a derivative of pBBR1MCS-5, harboring *GlpF* gene under control of glp1 promoter and *Gdh* gene under control of gdh promoter	This study
pBBR-P_{glp2}-GlpF-P_{gdh}-Gdh	Gm^R, a derivative of pBBR1MCS-5, harboring *GlpF* gene under control of glp2 promoter and *Gdh* gene under control of gdh promoter	This study

Table 2. As a blank control, we used the *G. oxydans* strain 621H containing the empty vector pBBR1MCS-5.

Shake Flask Fermentation of Strains

We cultured single colonies in a 250-mL flask containing 30 mL of sorbitol medium as the seed culture for 20 h at 30 °C, 220 r/min. Then, we inoculated a 5% (v/v) seed culture into the fermentation medium with different glycerol concentrations for 48 h at 30 °C, 220 r/min, and measured the OD600 of the fermentation broth using a UV–Vis spectrophotometer (Oppler, 752N, China). We used deionized water as a blank control.

HPLC Analysis of DHA

The fermentation broth was centrifuged at 8000 r/min for 10 min to obtain the supernatant, which was used for DHA measurement. We filtered the samples with a 0.22-μm filter membrane. To conduct high-performance liquid chromatography (HPLC), we used a Hypersil 5-NH2 (5 μm, 4.6 × 250 mm) HPLC column (Series III) from Agilent Technologies equipped with a refractive index detector and a column temperature of 30 °C. The volume of the injected sample was 20 μL. We used acetonitrile/H_2O ($v/v = 60$: 40) as the mobile phase at a flow rate of 1 mL/min. We determined the DHA concentration using a calibration curve for standard DHA solutions.

Determination of Enzyme Activity

We cultured wild-type and engineered strains in sorbitol medium for 24 h at 30 °C, 220 r/min. The broth was then centrifuged at 10,000*g* for 10 min to obtain cells, after which the cells were washed twice in a 50 mmol/L phosphate buffer (pH 7.0). After washing, the cells were suspended in 20 mL of 50 mmol/L phosphate buffer (pH 7.0). Then, these suspended cells were broken by an ultrasonic wave and subsequently kept in an ice bath. The broken cells were collected by centrifugation, and the glycerol dehydrogenase activity was determined according to the method reported by Sugisawa and Hoshino [37].

Using 2,6-dichlorophenolindophenol (DCIP) as the electron acceptor, we detected changes in the absorbance at 600 nm during the reaction to determine the glycerol dehydrogenase activity in the cell membrane fraction. The basal reaction solution included 50 mmol/L of potassium phosphate buffer (pH 6.0), 0.25 mmol/L of DCIP, and 0.325 mmol/L of phenazine methosulfate (PMS). The basal reaction solution was prepared just prior to the assay.

When measuring the enzyme activity, we added 2.4 mL of basal reaction solution and 30 μL of diluted crude enzyme

solution to the cuvette. After 5 min in a water bath at 30 °C, we added 600 μL of glycerol (200 mmol/L, 30 °C) to the mixture to start the reaction. We measured the results at 600 nm. The extinction coefficient of the DCIP at a pH of 6.0 was 10.8 mmol/L. In this study, we defined one enzyme unit as the amount of the enzyme that catalyzes a reduction of 1 mmol of DCIP per min when the pH is 6.0 at 30 °C. The formula we used to calculate the enzyme activity is as follows:

$$\rho = \frac{\left(\frac{\Delta A}{\Delta t}\right) \times \frac{1}{\varepsilon}}{C_{\mathrm{Pr}} \times V_{\mathrm{E}}} \quad (1)$$

where ΔA is the change in the absorption value at 600 nm in Δt (min); V_{E} is the volume of the enzyme solution to be measured; ε (mmol/L) is the extinction coefficient of DCIP; and C_{Pr} (mg/mL) is the protein concentration of the enzyme solution.

Results and Discussion

Overexpression of Glycerol Transporter Gene to Improve Cell Growth

We overexpressed *GlpF* using the promoters P_{tufB}, P_{gmr}, P_{glp1} and P_{glp2} to, respectively, construct the strains G1, G2, G3, and G4. Then, we cultured *G. oxydans* strains 621H, G1, G2, G3, and G4 in a sorbitol medium to study the effect of overexpressing the gene *GlpF*. Figure 1a shows the OD600 values of different strains, and we can see that the OD600 values of all engineered strains were much higher than that of the *G. oxydans* strain 621H. At 48 h, the OD600 values of strains G1, G2, G3, and G4 increased by 110.5%, 48.4%, 108.4%, and 72.6%, respectively, compared to that of the control group.

To further explain this phenomenon, we analyzed the *GlpF* transcript levels of the *G. oxydans* strains 621H, G1, G2, G3, and G4 after 48 h of incubation in sorbitol medium. As shown in Fig. 1b, the *GlpF* transcript levels in the engineered strains were all higher than that of the control group, especially in strains G1 and G3, which was consistent with the growth levels of these strains. These results show that overexpressing *GlpF* can effectively improve the growth of strains, especially when using the promoters P_{tufB} and P_{glp1}.

Overexpression of Glycerol Transporter Gene to Improve the Glycerol Tolerance

To investigate the tolerance of engineered strains to glycerol, we cultured the *G. oxydans* strains 621H, G1, G2, G3, and G4 in fermentation medium. Figure 2 shows the OD600 values of *G. oxydans* strains 621H, G1, G2, G3, and G4 in the fermentation medium with different glycerol concentrations, and we can see that the OD600 values of the engineered strains were all much higher than that of the *G. oxydans* strain 621H at the same glycerol

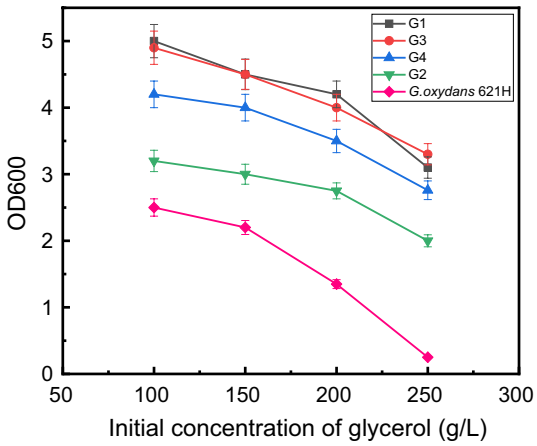

Fig. 2 OD600 values of *G. oxydans* strains 621H, G1, G2, G3, and G4 in fermentation medium with different concentrations of glycerol. *G. oxydans* strain 621H served as the control group. The *GlpF* gene was overexpressed in strains G1, G2, G3, and G4 by the use of promoters P_{tufB}, P_{gmr}, P_{glp1}, and P_{glp2}, respectively

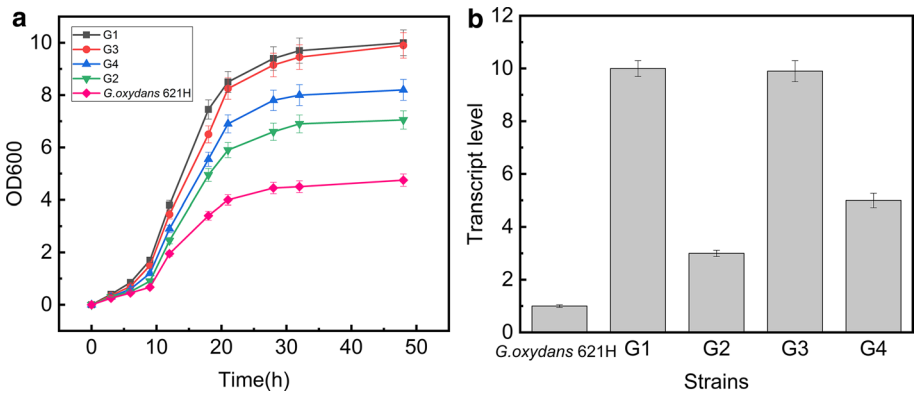

Fig. 1 **a** OD600 values of the *G. oxydans* strains 621H, G1, G2, G3, and G4 in sorbitol medium. **b** Relative *GlpF* transcript levels of the *G. oxydans* strains 621H, G1, G2, G3, and G4 in sorbitol medium. The *GlpF* transcript level in *G. oxydans* 621H was set to 1. *G. oxydans* strain 621H served as the control group. The *GlpF* gene was overexpressed in strains G1, G2, G3, and G4 by the use of promoters P_{tufB}, P_{gmr}, P_{glp1}, and P_{glp2}, respectively

concentrations. When the initial concentration of glycerol was 100 g/L, the OD600 values of strains G1, G2, G3, and G4 increased by 100%, 28%, 96%, and 68%, respectively, compared with that of the control group. At a glycerol concentration of 150 g/L, the OD600 values of strains G1, G2, G3, and G4 increased by 104.5%, 36.4%, 104.5%, and 81.8%, respectively, compared with that of the control group. When the initial glycerol concentration reached 200 g/L, the OD600 values of strains G1, G2, G3, and G4 increased by 211.1%, 103.7%, 196.3%, and 159.3%, respectively, compared with that of the control group. When the initial concentration of glycerol reached 250 g/L, the OD600 values of strains G1, G2, G3, and G4 increased by 1140%, 700%, 1220%, and 1004%, respectively, compared with that of the control group.

The results show that for the same strain, the growth of the strain was inhibited with an increase in the glycerol concentration. However, the inhibitory degree against different strains was different. The growth of *G. oxydans* 621H was more severely inhibited than strains overexpressing *GlpF*. Strains G1 and G3, overexpressing *GlpF* with the promoters P_{tufB} and P_{glp1}, respectively, performed better in improving glycerol tolerance. These results reveal that overexpressing *GlpF* can effectively improve the glycerol tolerance of strains, especially when using promoters P_{tufB} and P_{glp1}.

Then, we selected the most glycerol-tolerant strain G1 for DHA production with different glycerol concentrations. Figure 3a, b shows the contents of DHA and residual glycerol, respectively.

Figure 3a shows that *GlpF* overexpression did not enhance but in fact reduced the production of DHA. In Fig. 3b, we can see that the residual glycerol concentrations of *G. oxydans* 621H and G1 are similar. We suppose that some glycerol was converted into DHA by membrane-bound glycerol dehydrogenase in the periplasmic space of strain G1 without the need to enter the cytoplasm. In addition, *GlpF* overexpression improved the uptake rate of glycerol and caused more glycerol to be metabolized for cell growth after entering the cytoplasm than *G. oxydans* 621H. Therefore, although the production of DHA decreased, the residual glycerol concentration changed slightly and the cell growth was enhanced compared with that of *G. oxydans* 621H.

Overexpression of Glycerol Dehydrogenase Gene to Increase the DHA Titer by Shaking Flask Fermentation

We overexpressed *Gdh* using promoters P_{gdh} and P_{tufB}, respectively, to construct strains G5 and G6. We cultured *G. oxydans* strains 621H, G5, and G6 in 100-g/L glycerol fermentation medium. Figure 4a shows the OD600 values of different strains. In Fig. 4a, we can see that overexpressing *Gdh* did not have a significant effect on the growth of strains. The growth conditions of strains G5 and G6 were basically

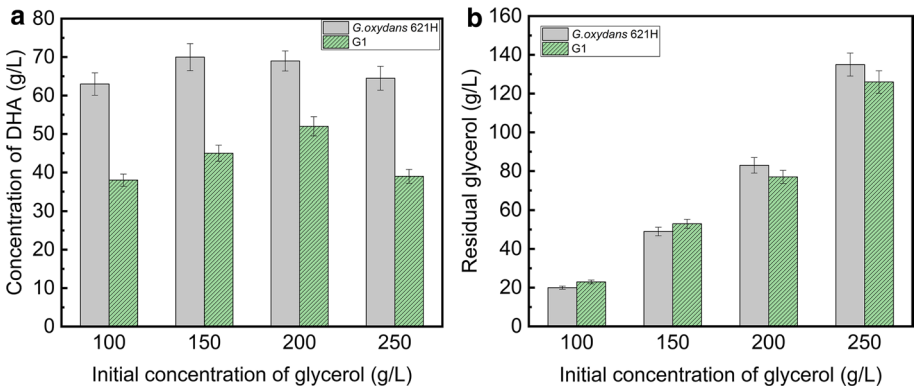

Fig. 3 **a** Concentration of DHA produced by *G. oxydans* strains 621H and G1 in fermentation medium. **b** Residual glycerol concentrations of *G. oxydans* strains 621H and G1 in fermentation medium. *G. oxydans* strain 621H served as the control group. The *GlpF* gene was overexpressed in G1 by the use of promoter P_{tufB}

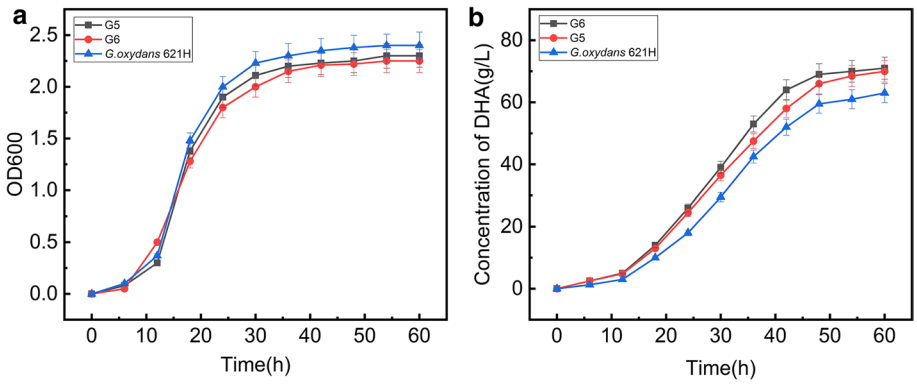

Fig. 4 **a** OD600 values of *G. oxydans* strains 621H, G5, and G6 in 100-g/L glycerol fermentation medium. **b** DHA titer produced by *G. oxydans* strains 621H, G5, and G6 in 100-g/L glycerol fermentation medium. *G. oxydans* strain 621H served as the control group. We overexpressed the *Gdh* gene in strains G5 and G6 by the use of promoters P_{gdh} and P_{tufB}, respectively

the same as that of *G. oxydans* strain 621H. At 27 h, the growth of *G. oxydans* strains 621H, G5, and G6 all reached the stationary phase. At 60 h, their OD600 values were 2.40, 2.30, and 2.25, respectively.

To quantify the fermentation products, we used the authentic standards curve. Figure 4b shows the DHA produced by different strains. In the figure, we can see that at the beginning of the reaction, the DHA titer increased slowly and then accelerated. During the late stages of fermentation, it increased gradually and reached the stationary phase after 48 h.

The DHA titers produced by engineered strains under the control of promoters P_{gdh} and P_{tufB} were all higher than that of *G. oxydans* strain 621H. After fermentation, the DHA titers produced by strains G5 and G6 were about 8 g/L higher than that of *G. oxydans* strain 621H, which increased by almost 12.7%. This indicates that overexpressing glycerol dehydrogenase in *G. oxydans* 621H can increase the DHA titer. Li et al. [38] constructed an engineered strain for the industrial production of DHA by overexpressing the *Gdh* gene in *G. oxydans* M5AM, in which the gene coding for the membrane-bound alcohol dehydrogenase (*Adh*) is interrupted. The DHA titer in that study was 96 g/L from 100 g/L glycerol, which is higher than that obtained in our study. This may be due to the absence of the *Adh* gene in the *G. oxydans* strain M5AM.

Transcript Level and Glycerol Dehydrogenase Activity in Strains Overexpressing Glycerol Dehydrogenase Gene

Next, we cultured the *G. oxydans* strains 621H, G5, and G6 in a 100-g/L glycerol fermentation medium for 48 h and then analyzed the transcript levels of the *Gdh* gene, the results of which are shown in Fig. 5a. In the figure, we can see that all the transcript levels of the engineered strains under the control of the promoters P_{gdh} and P_{tufB} were about four times higher than that of *G. oxydans* 621H. Glycerol dehydrogenase is the key enzyme responsible for the synthesis of DHA by the catalysis of glycerol in *G. oxydans* 621H. The difference in the transcript levels of the *Gdh* gene in the engineered and wild-type strains explains the difference in the DHA production in Fig. 4b.

To further explain the increase in the DHA production, we measured the activity of glycerol dehydrogenase in *G. oxydans* strains 621H, G5, and G6, the results of which are shown in Fig. 5b. In the figure, we can see that the activity of glycerol dehydrogenase controlled by the P_{gdh} promoter increased from 1.74 to 2.16 U/mg, and the activity of glycerol dehydrogenase controlled by the P_{tufB} promoter increased from 1.74 to 2.25 U/mg. The activities of glycerol dehydrogenase in strains G5 and G6 were higher than those in the *G. oxydans* strain 621H, which could also explain the increase in the DHA production.

Co-overexpression of Glycerol Transporter and Glycerol Dehydrogenase Genes to Produce DHA by Shaking Flask Fermentation

Overexpressing *GlpF* can effectively improve the glycerol tolerance of strains, and overexpressing *Gdh* in *G. oxydans* can increase the DHA titer. As such, we attempted to overexpress *GlpF* and *Gdh* in *G. oxydans* simultaneously. We linked *GlpF* with promoters P_{tufB}, P_{gmr}, P_{glp1}, and P_{glp2}, respectively, and linked *Gdh* with the promoters P_{tufB} and P_{gdh}, respectively. Then, they were combined to construct strains G7, G8, G9, G10, G11, G12, G13, and G14. All these strains were cultured in 100-g/L glycerol fermentation medium. After 48 h, we identified and recorded the fermentation products by HPLC. Figure 6a

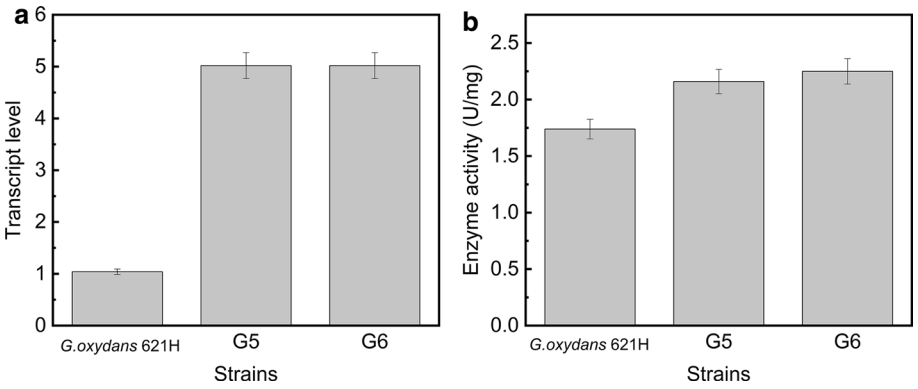

Fig. 5 **a** Relative transcript levels of the *Gdh* gene in *G. oxydans* strains 621H, G5, and G6 in 100-g/L glycerol fermentation medium. The transcript level of the *Gdh* gene in *G. oxydans* 621H was set to 1. **b** Enzyme activities of glycerol dehydrogenase in *G. oxydans* strains 621H, G5, and G6 in 100-g/L glycerol fermentation medium. *G. oxydans* strain 621H served as the control group. The *Gdh* gene was overexpressed in strains G5 and G6 by the use of the promoters P_{gdh} and P_{tufB}, respectively

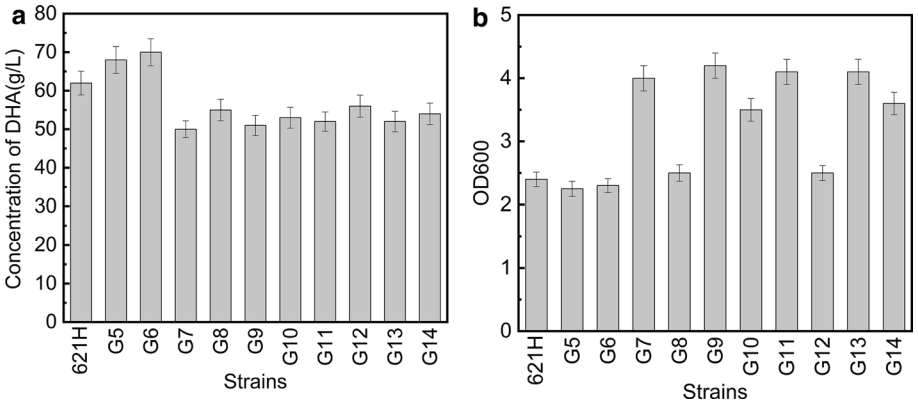

Fig. 6 **a** DHA titers produced by *G. oxydans* strains 621H, G5, G6, G7, G8, G9, G10, G11, G12, G13, and G14 in 100-g/L glycerol fermentation medium. **b** OD600 values of *G. oxydans* strains 621H, G5, G6, G7, G8, G9, G10, G11, G12, G13, and G14 in 100-g/L glycerol fermentation medium. The term 621H indicates the *G. oxydans* strain 621H, which served as the control group. The *Gdh* gene was overexpressed in strains G5 and G6 by the use of promoters P_{gdh} and P_{tufB}, respectively. The *GlpF* and *Gdh* genes were co-overexpressed in strains G7, G8, G9, G10, G11, G12, G13, and G14

shows the DHA titers produced by different strains, and Fig. 6b shows the OD600 values of these strains.

As shown in Fig. 6, the DHA titers of strains G7, G8, G9, G10, G11, G12, G13, and G14 are less than those in *G. oxydans* strains 621H, G5, and G6. However, the OD600 values of strains G7, G8, G9, G10, G11, G12, G13, and G14 were all higher than those of *G. oxydans* strains 621H, G5, and G6. Although the growth of strains co-overexpressing *GlpF* and *Gdh* was successfully enhanced, the DHA titers were reduced, which indicates that co-overexpressing *GlpF* and *Gdh* does not improve DHA production.

Transcript Level and Activity of Glycerol Dehydrogenase in Strains Co-overexpressing Glycerol Transporter Gene and Glycerol Dehydrogenase Gene

We measured the transcript levels of the *Gdh* gene in engineered strains, the results of which are shown in Fig. 7a. In the figure, we can see that the *Gdh* gene transcript levels in strains G7, G8, G9, G10, G11, G12, G13, and G14 and strains G5 and G6 are similar. Both are about five times the transcript level of *G. oxydans* strain 621H, which indicates that the overexpression of the glycerol transporter did not affect the transcript level of glycerol dehydrogenase.

Next, we measured the activity of glycerol dehydrogenase in the engineered strains, the results of which are shown in

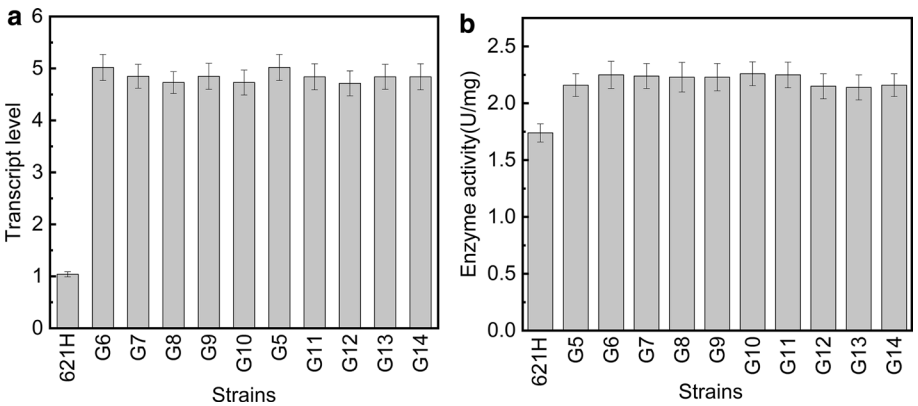

Fig. 7 **a** Relative transcript levels of *Gdh* in *G. oxydans* strains 621H, G5, G6, G7, G8, G9, G10, G11, G12, G13 and G14 in 100-g/L glycerol fermentation medium. The *Gdh* transcript level in *G. oxydans 621H* was set to 1. **b** Enzyme activities of *G. oxydans* strains 621H, G5, G6, G7, G8, G9, G10, G11, G12, G13, and G14 in 100-g/L glycerol fermentation medium. The term 621H indicates *G. oxydans* strain 621H, which served as the control group. The *Gdh* gene was overexpressed in strains G5 and G6 by the use of promoters P_{gdh} and P_{tufB}, respectively. *GlpF* and *Gdh* were co-overexpressed in strains G7, G8, G9, G10, G11, G12, G13, and G14

Fig. 7b. In the figure, we can see that the overexpression of *GlpF* had very little effect on the enzyme activity of glycerol dehydrogenase.

The effect of co-overexpressing *GlpF* and *Gdh* in *G. oxydans* was not as good as we had expected. High *Gdh* transcript levels and high enzyme activities of glycerol dehydrogenase in strains co-overexpressing *GlpF* and *Gdh* did not lead to high DHA titers. This indicates that although overexpressing *GlpF* can enhance cell growth and improve the glycerol tolerance of *G. oxydans*, it had negative effects on DHA production. We assume that *GlpF* overexpression improved the uptake rate of glycerol and caused more glycerol to be metabolized for cell growth after entering the cytoplasm than strains not overexpressing *GlpF*. The more glycerol that enters the cytoplasm for cell growth, the less glycerol is converted into DHA by the membrane-bound glycerol dehydrogenase in the periplasmic space. Therefore, the production of DHA decreased.

Conclusions

Our results indicate that overexpressing *GlpF* alone effectively promotes the growth of cells and improves the glycerol tolerance of *G. oxydans*, but it reduces the synthesis of DHA. Overexpressing *Gdh* alone increases the DHA titer by 12.7% from that of its parental strain. However, co-overexpressing *GlpF* and *Gdh* does not achieve a good balance between enhancing cell growth and increasing DHA production. Although it enhances cell growth, it fails to increase the DHA titer.

Future research should focus on analyzing the role of the glycerol transporter in DHA synthesis using transcriptomics and metabolomics methods and searching for new targets with which to construct recombinant strains to produce DHA at high glycerol concentrations.

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