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Combining metabolic engineering and adaptive evolution to enhance the production of dihydroxyacetone from glycerol by *Gluconobacter oxydans* in a low-cost way

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ABSTRACT

Gluconobacter oxydans can rapidly and effectively transform glycerol to dihydroxyacetone (DHA) by membrane-bound quinoprotein sorbitol dehydrogenase (mSLDH). Two mutant strains of GDHE Δ*adh* pBBR-P_{tufB}*sldAB* and GDHE Δ*adh* pBBR-*sldAB* derived from the GDHE strain were constructed for the enhancement of DHA production. Growth performances of both strains were largely improved after adaptively growing in the medium with glucose as the sole carbon source. The resulting GAT and GAN strains exhibited better catalytic property than the GDHE strain in the presence of a high concentration of glycerol. All strains of GDHE, GAT and GAN cultivated on glucose showed enhanced catalytic capacity than those grown on sorbitol, indicating a favorable prospect of using glucose as carbon source to reduce the cost in industrial production. It was also the first time to reveal that the expression level of the *sldAB* gene in glucose-growing strains were higher than that of the strains cultivated on sorbitol.

1. Introduction

Dihydroxyacetone (DHA) is one of important chemical and biochemical materials, which is used as sunless tanning agent, pharmaceutical precursors and functional additives (Claret et al., 1994; Hekmat et al., 2003). The most popular industrial form of producing DHA is the biotransformation of glycerol to DHA with *Acetobacter* species, *Gluconobacter* species and *Yeast* species, especially *Gluconobacter oxydans*. The microbial processes were preferred because of their specific nature and the mild process environment. In the past, great efforts had been devoted to the screening of excellent strains, optimization of medium composition and fermentation parameters (Bauer et al., 2005; Fidaleo et al., 2006; Hekmat et al., 2003; Wei et al., 2007, 2009; Wethmar and Deckwer, 1999). Recent advances in genetic manipulation have allowed the elimination of product or substrate inhibition and the improvement of DHA production. It was reported that the overexpression of the *sldAB* gene that encodes membrane-bound sorbitol dehydrogenase (mSLDH) could obviously enhance the production of DHA (Gatgens et al., 2007; Li et al., 2010). Recent evidence has also shown that the growth of *G. oxydans* in a high concentration of glycerol was largely improved when the membrane-bound alcohol dehydrogenase (ADH) (encoded by the *adh* gene) was deficient (Habe et al., 2009a, 2009b, 2010; Li et al., 2010).

Glucose is the favorable carbon source for many microorganisms, but not for *G. oxydans* which prefers to use glycerol, sorbitol or mannitol. When glucose is used as carbon source, only a minor part of glucose is assimilated in the cytoplasm by the $NADP^+$-dependent soluble glucose dehydrogenase (sGDH) and further dissimilated via the pentose phosphate (PP) pathway or the Entner-Doudoroff (ED) pathway (Holscher et al., 2009; Olijve and Kok, 1979; Rauch et al., 2010). The majority of glucose is directly oxidized to gluconate by membrane-bound pyrroloquinoline quinone (PQQ)-dependent glucose dehydrogenase (mGDH, encoded by the *mgdh* gene) in the periplasm space. The obtained gluconate is further oxidized to 2-ketogluconate, 2,5-diketogluconate as well as 5-ketogluconate, which are then secreted almost completely into the medium, causing extremely acidic environment and therefore significant inhibition of cell growth (Gupta et al., 2001; Holscher and Gorisch, 2006; Holscher et al., 2009). In addition, the dissolved oxygen was mainly consumed for the oxidation process of glucose by mGDH, resulting in severe oxygen limitation on the growth of the wild-type strain in glucose medium (Silberbach et al., 2003).

In industrial production, it is preferable to obtain large amount of *G. oxydans* cells as biocatalyst on relatively cheap carbon source. It was indicated that mutants deficient in mGDH could exhibit

Abbreviations: ADH, membrane-bound alcohol dehydrogenase; C_{max}, maximum cell density; DHA, dihydroxyacetone; HPLC, high performance liquid chromatography; mGDH, pyrroloquinoline quinone (PQQ)-dependent glucose dehydrogenase; mSLDH, membrane-bound quinoprotein sorbitol dehydrogenase; PCR, polymerase chain reaction; PQQ, pyrroloquinoline quinine; qRT-PCR, quantitative real-time PCR; RT-PCR, reverse transcription PCR; sGDH, $NADP^+$-dependent soluble glucose dehydrogenase; μ_{max}, maximum specific growth rate.

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enhanced growth rate and biomass yield on glucose (Gupta et al., 1997; Krajewski et al., 2010; Zhu et al., 2011). However, less attention has been paid to the integration of biotransformation performance and growth improvement of the mutant strains in glucose medium. In this work, the previously constructed *G. oxydans* GDHE strain that was deficient in membrane-bound glucose dehydrogenase and adaptively evolved on glucose was further modified by knocking out the gene encoding alcohol dehydrogenase and overexpressing the sorbitol dehydrogenase gene with different promoters. The growth performances of the engineered strains were adaptively evolved and the biotransformation of glycerol into DHA by different strains was investigated under various conditions.

2. Methods

2.1. Strains, plasmids, and growth conditions

The strains and plasmids used in this study were summarized in Table 1. *G. oxydans* strains were cultivated in sorbitol medium (sorbitol 80 g/L, yeast extract 20 g/L, KH_2PO_4 1.5 g/L, $(NH_4)_2SO_4$ 1.5 g/L and $MgSO_4{\cdot}7H_2O$ 0.5 g/L) or glucose medium (glucose 20 g/L, yeast extract 20 g/L, KH_2PO_4 1.5 g/L, $(NH_4)_2SO_4$ 1.5 g/L and $MgSO_4{\cdot}7H_2O$ 0.5 g/L), at 30 °C, 200 rpm. Sorbitol medium or glucose medium without salt solution (KH_2PO_4 1.5 g/L, $(NH_4)_2SO_4$ 1.5 g/L and $MgSO_4{\cdot}7H_2O$ 0.5 g/L) were used to examine the effect of salt solution on biotransformation of glycerol to DHA. *E. coli* JM109 and *E. coli* HB101 were cultivated at 37 °C, 200 rpm on Luria–Bertani medium (yeast extract 5 g/L, tryptone 10 g/L, NaCl 10 g/L) with appropriate antibiotics.

2.2. General genetic techniques

Primers used in this study were listed in Table 2. DNA manipulation was according to the standard protocols. For PCRs, genomic DNA isolated from *G. oxydans* 621H (DSM 2343) was used as a template. *LA* Taq polymerase (TaKaRa Biotechnol., Dalian, China) was used for PCR amplification in test reactions and reverse transcription PCR (RT-PCR).

Table 2
Primers used in this study.

Primer	Sequence (5′–3′)
tufB-fwd	CGATGGTAAGAGCTCCACTGCCG
tufB-rev	CCCCGCTCTAGATGGAACGGG
sldAB-fwd-N	CGCTCTAGAACAACACCTGGTTCTGGAT
sldAB-fwd	GCGTCTAGACTTTCAGTTCTGGAGGCTTTCA
sldAB-rev	GCTTCCCACCCGAATTCTGGAAAAAACG
Amp-*Nco*I	CTCCCATGGATGAGTAAACTTGGTCTGA
Amp-*Bgl*II	TACAGATCTTGACGGGCTTGTCTGCTC
ADH-fwd	ACTTCTGGTCTACTGAC
ADH-rev	TCTCAGATACCAGCCTG
adh-*Xba*I-fwd	CTATCTAGAACATACTGGACCGCTCAT
adh-*Eco*RI-rev	TCAGAATTCTGCCCTGCGGACAGACAT
sldAB-RT-fwd	CCTGCGTAGCCCTGAAGAAAAC
sldAB-RT-rev	CGAGCCGATGTCATAGTCCC
16S-RT-fwd	GCGGTTGTTACAGTCAGATG
16S-RT-rev	GCCTCAGCGTCAGTATCG

2.3. Construction of expression vector

Knockout vector pSUP202-*adhA*::Km was kindly provided as a gift from the Institute of Newworld Biotechnology (Wei et al., 2010). Based on the available sequence information for *G. oxydans* 621H (Prust et al., 2005), the promoter *tufB*, gene *sldAB* and gene *sldAB* with its native promoter were amplified by PCR with primers *tufB*-fwd and *tufB*-rev, *sldAB*-fwd and *sldAB*-rev, *sldAB*-fwd-N and *sldAB*-rev, respectively (Table 2). The resulting fragments *tufB* digested with restriction enzymes *Sac*I and *Xba*I (Fermentas, EU), and gene *sldAB* digested with restriction enzymes *Xba*I and *Eco*RI (Fermentas, EU) were successively ligated into the prepared vector pBBR1MCS4 using T4 DNA ligase (Fermentas, EU), generating plasmid pBBR-P_{tufB} and pBBR-P_{tufB}*sldAB* respectively. PCR products *sldAB* with its native promoter were digested with restriction enzymes *Xba*I and *Eco*RI and ligated into the prepared vector pBBR1MCS4 using T4 DNA ligase, resulting in plasmid pBBR-*sldAB*. The above procedures were showed in Fig. 1. The recombinant plasmids were transformed into *E. coli* JM109 for storage. Transformants were selected on LB agar plates containing 10 μg/mL ampicillin, and further confirmed by colony PCR and restriction enzyme digestions.

Table 1
Strains and plasmids used in this study.

Strains or plasmids	Relevant properties	Reference or source
Strains		
G. oxydans strains		
621H (DSM 2343)		Purchased from DSMZ
GDHK	Gm^r; *G. oxydans* 621H derivative, *mgdh*::Gm^r	Zhu et al. (2011)
GDHE	Gm^r; *G. oxydans* 621H derivative, *mgdh*::Gm^r, after 50 days evolution	This study
GDHE Δ*adh*	Gm^r; Km^r; GDHE derivative, *mgdh*::Gm^r; *adh*::Km^r	This study
GDHE Δ*adh* pBBR-P_{tufB}*sldAB*	Gm^r; Km^r; Am^r; GDHE Δ*adh* derivative, *mgdh*::Gm^r; *adh*::Km^r; pBBR-P_{tufB}*sldAB*	This study
GDHE Δ*adh* pBBR-*sldAB*	Gm^r; Km^r; Am^r; GDHE Δ*adh* derivative, *mgdh*::Gm^r; *adh*::Km^r; pBBR-*sldAB*	This study
GAT	Gm^r; Km^r; Am^r; GDHE Δ*adh* pBBR-P_{tufB}*sldAB* derivative, after 25 days evolution, *mgdh*::Gm^r; *adh*::Km^r; pBBR-P_{tufB}*sldAB*	This study
GAN	Gm^r; Km^r; Am^r; GDHE Δ*adh* pBBR-*sldAB* derivative, after 25 days evolution, *mgdh*::Gm^r; *adh*::Km^r; pBBR-*sldAB*	This study
E. coli strains		
E. coli JM109	*recA1*, *endA1*, *gyrA96*, *thi*, *hsdR17*, *supE44*, *relA1*, *Δ(lac-proAB)*/F[*traD36*, *proab*$^+$, *lacI*^{q}, *lacZ*ΔM15]	
E. coli HB101	F^-, *hsdS20*, *recA13*, *ara-14*, *proA2*, *lacY1*, *galK2*, *rpsL20(str)*, *xyl-5*, *mtl-1*, *sup E44*, *leuB6*, *thi-1*	
Plasmids		
pRK2013	Km^r; helper plasmid for triparental mating	ATCC
pSUP202	Am^r; Tc^r; Cm^r; mob	
pGEM-3Zf	Am^r; used as template of ampicillin gene cassette	
pSUP202-3-adhA::Km	Km^r; *adh*::Km^r	Wei et al. (2010)
pBBR1MCS4	Am^r; *lacZ* P_{lac}; pBBR1 replicon	Kovach et al. (1994)
pBBR-P_{tufB}	Am^r; pBBR1MCS4 carrying promoter *tufB*	This study
pBBR-P_{tufB}*sldAB*	Am^r; pBBR1MCS4 carrying *sldAB* with promoter *tufB*	This study
pBBR-*sldAB*	Am^r; pBBR1MCS4 carrying *sldAB* with its native promoter	This study

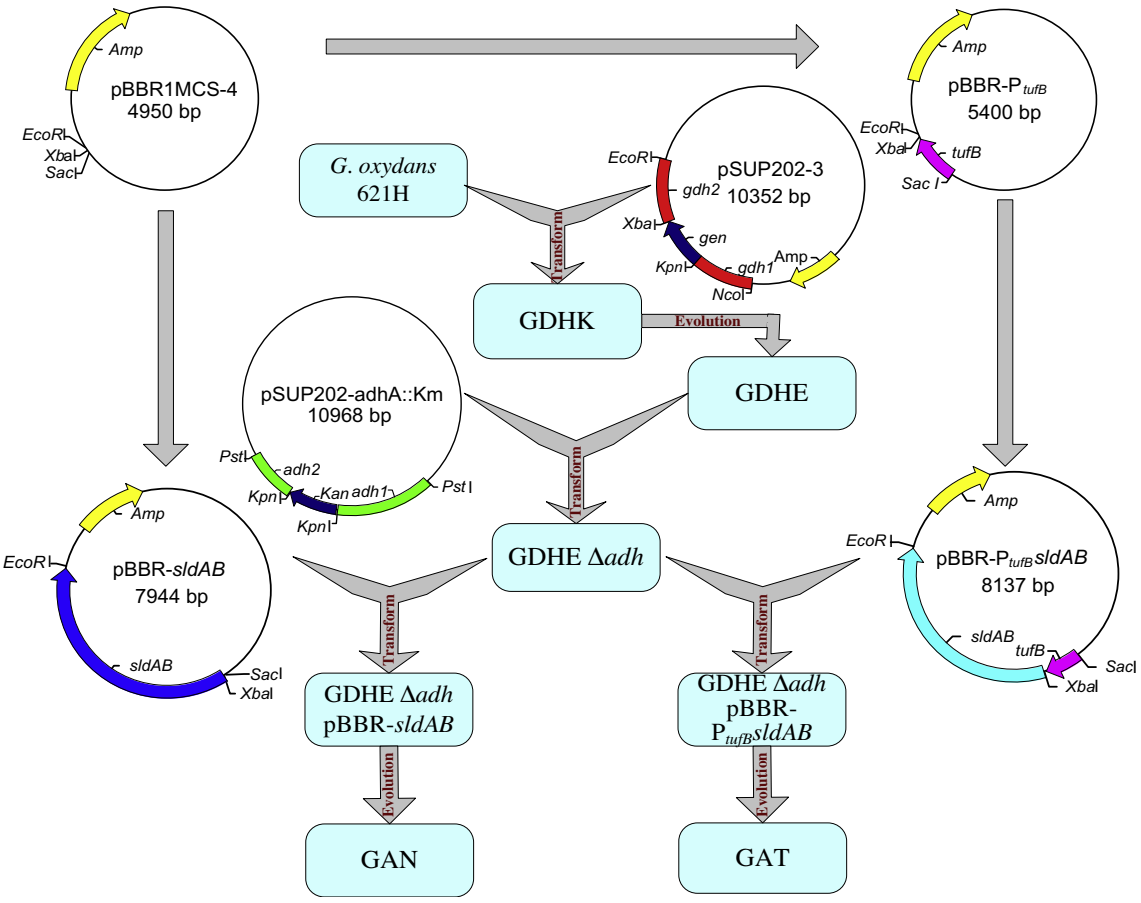

Fig. 1. Plasmids and strains construction.

2.4. Conjugational plasmid transfer into G. oxydans

Knockout vector pSUP202-*adhA*::Km, expression vector pBBR-P_{tufB}*sldAB* and expression vector pBBR-*sldAB* were transferred into corresponding *G. oxydans* strains by triparental mating in order as shown in Fig. 1 (Holscher and Gorisch, 2006). *E. coli* JM109 was used to bear corresponding vectors as the donor strain and *E. coli* HB101 was used to harbor mobilizing plasmid pRK2013 as the helper strain. Three strains were grown to the late-exponential phase, pelleted, washed, resuspended in sorbitol medium, and mixed at a 1:1:1 ratio. The mixture was spread on sorbitol agar plates without antibiotics and incubated overnight at 30 °C. The grown cell patches were scraped from the plates and resuspended in sterile water, then streaked on selective sorbitol agar plate containing appropriate selective antibiotic (gentamycin, kanamycin or ampicillin). Plates were incubated for 2 to 4 days at 30 °C until resistant colonies appeared. Extracting genomic DNA or plasmids of these resistant colonies, polymerase chain reaction, direct sequencing or restriction enzyme reaction were performed to remove false positives and identify the objective mutations.

2.5. Laboratory adaptive evolution

Cell growth was evaluated by spectrophotometric measurements at 600 nm using a spectrophotometer (UV1800, Shimadzu, Kyoto, Japan). The growth of strains was characterized by maximum specific growth rate (μ_{max}). Adaptive evolution experiments were started from individual colonies and were conducted in triplicate in 250 mL Erlenmeyer flasks containing 50 mL of glucose medium with appropriate antibiotics at 200 rpm and 30 °C. Throughout the course of adaptive growth, serial transfers were made during the mid-exponential growth phase. The level of dilution at each passage was adjusted regularly to allow transferring strains to fresh medium at 24-h intervals. Cultures were frozen and stored at regular intervals, and the adaptive evolution experiments were performed until stable μ_{max} was achieved. Glucose concentration in the medium was determined enzymatically with microplate spectrofluorometer (PowerWave XS/XS2, BioTek Instruments, Winooski, VT). The concentration of D-sorbitol was analyzed using an HPLC equipped with a refractive index detector and a GL Insertsil NH_2 analysis column (4.6 mm by 250 mm). Acetonitrile-H_2O (82:18, v/v) was used as eluent with a flow rate of 1.0 mL/min at 80 °C.

2.6. Detection of gene expression by quantitative real-time PCR (qRT-PCR)

For quantitative real-time PCR experiments, *G. oxydans* strains were grown to late-exponential phase in sorbitol medium or glucose medium. Total RNA was isolated using Trizol (Molecular Research Center, Cincinnati, OH) by following manufacturer's procedure. To remove residual DNA, total RNA was treated with DNase I for 30 min at 37 °C. RNA samples were reverse-transcribed with RevertAid™ First Stand cDNA Synthesis Kit (Fermentas, Burlington, Canada) according to the manufacturer's instructions. Subsequently, the quantitative gene analysis was performed with the Maxima™ SYBR Green qPCR Master MIX (2×) regent (Fermentas) on an Eppendorf Mastercycler Realplex^2 (Eppendorf AG, Hamburg, Germany), using oligonucleotides *sldAB*-RT-fwd as a forward primer and *sldAB*-RT-rev as a reverse primer. The 16S rRNA gene

was used as internal standard, which was obtained using a forward primer 16S-RT-fwd and a reverse primer 16S-RT-rev. The PCR conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 47.3 °C for 30 s, and 72 °C for 15 s. The $2^{-\Delta\Delta Ct}$ method was applied to analyze qRT-PCR data.

2.7. Whole-cell biotransformation of glycerol to DHA

The *G. oxydans* cells cultivated in different mediums in late-exponential phase were harvested by centrifugation (12,000 rpm, 10 min, 4 °C), washed three times with physiological saline (0.9% NaCl solution), and then resuspended in 0.1 M phosphate buffer (pH 6.0). Bioconversions were carried out in triplicate in 50 mL shake flasks, containing 1.0 g CDW/L cells and 10 g/L, 40 g/L, or 100 g/L glycerol at 200 rpm and 30 °C. Samples (1 mL) were taken at suitable intervals, and the supernatants were obtained after centrifuging at 12,000 rpm and 4 °C for 10 min. Reaction samples were taken every 20 min during the first 2 h to test specific catalytic activity.

2.8. Quantification of glycerol and DHA

The concentration of DHA was determined by HPLC, equipped with ZORBAX SB-AQ column (Agilent Technologies, Santa Clara, CA). The mobile phase was 0.1% H_3PO_4 with flow rate of 1 mL/min and the column temperature was maintained at 30 °C. During the analysis, the organic acid peaks were monitored by UV absorbance at 210 nm. Triglycerides reagent kit (Kexin Biotechnol., Shanghai, China) was used to quantify the amount of glycerol in the reaction system.

3. Results and discussion

3.1. Construction of various G. oxydans strains

In our previous study, a mutant strain GDHE that could grow well on glucose was obtained by deletion of the *mgdh* gene and subsequent adaptive growth with glucose as the sole carbon source (Zhu et al., 2011). It was proved that the deletion of the *adh* gene encoding membrane-bound alcohol dehydrogenase (ADH) could prevent the strain from the accumulation of glyceric acid and thus be advantageous to DHA production as well as the subsequent purification (Habe et al., 2009a, 2009b, 2010). Therefore, the *adh* gene of the GDHE strain was knocked out using the vector pSUP202-*adhA*::Km to obtain the GDHE Δ*adh* strain. Since overexpression of the *sldAB* gene was also reported to be advantageous to the biotransformation of glycerol to DHA (Gatgens et al., 2007), this gene in the GDHE Δ*adh* strain was overexpressed with expression vectors pBBR-P_{tufB}*sldAB* and pBBR-*sldAB*, respectively (Fig. 1). The growth of the resulting mutants GDHE Δ*adh* pBBR-P_{tufB}*sldAB* and GDHE Δ*adh* pBBR-*sldAB* were, however, significantly attenuated compared to the GDHE or GDHE Δ*adh* strains. It might be attributed to the accumulation of a large amount of gratuitous proteins, which was widely observed in cases of high-level induction of a recombinant protein or pathway (Kurland and Dong, 1996). To enhance the growth of GDHE Δ*adh* pBBR-P_{tufB}*sldAB* and GDHE Δ*adh* pBBR-*sldAB* on glucose, laboratory adaptive evolution was carried out for both strains with glucose as the sole carbon source. Similar to the evolutionary growth of the GDHK strain previously done (Fig. 2a), the maximum specific growth rates of both strains almost doubled at the end of evolution (Fig. 2b). Interestingly, the evolutionary processes for these two strains were remarkably faster (about 25 days) than that of the GDHK strain (approximately 50 days).

3.2. Growth profiles of various G. oxydans strains

Two constructed strains (GDHE Δ*adh* pBBR-P_{tufB}*sldAB* and GDHE Δ*adh* pBBR-*sldAB*, respectively), their evolved strains (GAT and GAN, respectively), and the GDHE strain (evolved on glucose from GDHK) were cultivated on either sorbitol or glucose to study their growth characteristics (Table 3). Though still slightly below those obtained on sorbitol, both specific growth rate and maximum cell density of the GAT and GAN strains were significantly improved in glucose medium. Additional experimental data showed that the growth phenotype of the GAN strain on glucose could not be enhanced further by higher glucose concentration (data not shown), suggesting the limited glucose catabolism for these strains. There may be various reasons for the impairment of glucose metabolism. First, although the transporter systems for the uptake of glucose and ketogluconates are still remain unknown, it has been suggested that most glucose-transporting PTS genes are absent in *G. oxydans* (Prust et al., 2005). *G. oxydans* has a biphasic growth behavior with glucose as the sole carbon source. In the first growth phase, glucose is rapidly but incompletely oxidized to gluconate and ketogluconates in the periplasm by membrane-bound dehydrogenases, which involves non-phosphorylative oxidation that channels electrons into the respiratory chain. Above mentioned products are then most likely taken up and further metabolized by enzymes of the pentose phosphate pathway in the following growth phase (KulhAnek, 1989; Olijve and Kok, 1979). The growth rate of *G. oxydans* in the second growth phase was reported to be lower compared to that in the first growth phase as energy generation from direct oxidation pathway was more efficient than that from the PP pathway (Olijve and Kok, 1979; Weenk et al., 1984). In addition, the incomplete glycolytic pathway and tricarboxylic acid cycle in *G. oxydans* limit the supply of NADH as electron donor for electron transport phosphorylation and thus cause the limited energy generation.

The biomass yields on glucose, however, did not increase too much during the course of adaptive growth, indicating additional genetic manipulation of genes relating to intracellular glucose metabolism might be required. For all strains studied, the biomass yields on glucose (0.14–0.17 gDCW/g) were more than 10-fold greater than those on sorbitol (0.011–0.014 gDCW/g).

Laboratory adaptive evolution has been proved a feasible and efficient technique to achieve improved cellular properties without requiring metabolic or regulatory details of the strain (Fong and Palsson, 2004). In this study, the maximum specific growth rates of the evolved strains (GAN and GAT) were doubled compared to the starting strains (GDHE Δ*adh* pBBR-P_{tufB}*sldAB* and GDHE Δ*adh* pBBR-*sldAB*) after adaptively growing on glucose (Table 3). Adaptive evolution allows one to investigate systematically the genetic and phenotypic changes of strains in the controlled environment and thus suggest efficient ways to improve strain properties. Based on the metabolic models and stable isotope-based labeling experiments, as already applied to several *E. coli* strains, the key factors or rate-limiting reaction steps for glucose utilization or biotransformation performance of the GAN and GAT strains might be successfully determined (Fong et al., 2006; Hua et al., 2006).

3.3. Biotransformation of glycerol to DHA by various G. oxydans mutants

Biotransformations of glycerol to dihydroxyacetone by mSLDH with the adaptively evolved strains GDHE, GAT and GAN cultivated on different carbon sources were investigated. Fig. 3 showed that overall conversion rates of glycerol to DHA by resting GAT or GAN cells harvested from glucose medium were obviously faster than those by sorbitol-grown resting cells. Detailed kinetic studies were also performed with both glucose-grown and sorbitol-grown

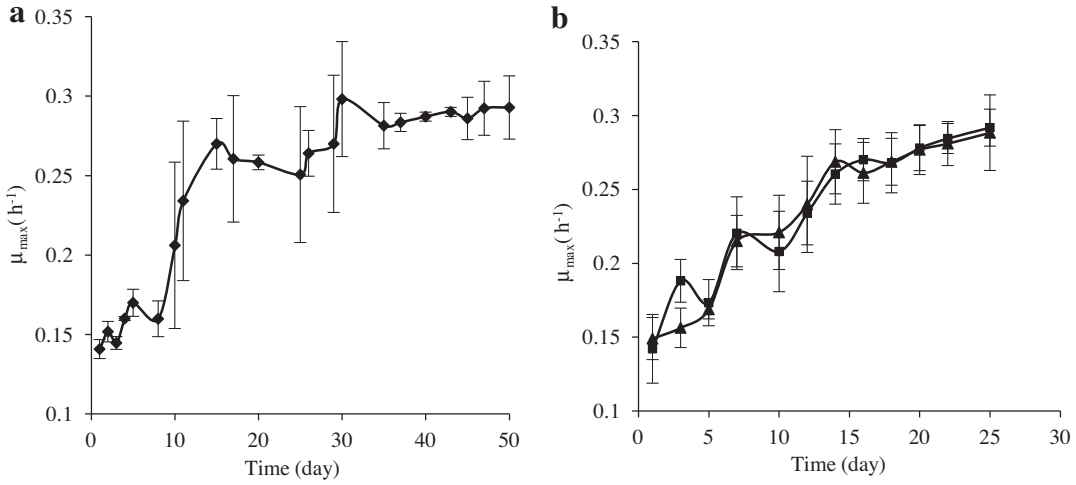

Fig. 2. Maximal specific growth rate (μ_{max}) of *G. oxydans* mutants during laboratory adaptive evolution on glucose. (a) Adaptive growth of GDHK (diamonds) to obtain GDHE. (b) Adaptive growth of GDHE Δ*adh* pBBR-P_{tufB}*sldAB* (triangles) and GDHE Δ*adh* pBBR-*sldAB* (squares) to obtain GAT and GAN, respectively.

Table 3
Growth kinetic parameters for various *G. oxydans* strains used sorbitol or glucose as sole carbon source.

Strain	80 g/L Sorbitol			20 g/L Glucose		
	μ_{max} (h^{-1})	C_{max} (gCDW/L)	$Y_{x/s}$ (gCDW/g)	μ_{max} (h^{-1})	C_{max} (gCDW/L)	$Y_{x/s}$ (gCDW/g)
GDHE	0.33 ± 0.02	1.12 ± 0.01	0.01 ± 0.00	0.29 ± 0.02	0.98 ± 0.02	0.14 ± 0.02
GDHE Δ*adh* pBBR-P_{tufB}*sldAB*	0.28 ± 0.02	1.03 ± 0.02	0.01 ± 0.00	0.15 ± 0.01	0.56 ± 0.02	0.14 ± 0.02
GDHE Δ*adh* pBBR-*sldAB*	0.28 ± 0.01	1.05 ± 0.03	0.01 ± 0.00	0.14 ± 0.01	0.56 ± 0.01	0.15 + 0.03
GAT	0.32 ± 0.02	1.22 ± 0.02	0.01 ± 0.01	0.28 ± 0.02	0.99 ± 0.02	0.16 ± 0.01
GAN	0.34 ± 0.01	1.19 ± 0.01	0.01 ± 0.01	0.29 ± 0.03	0.97 ± 0.04	0.17 ± 0.03

G. oxydans cells under various initial glycerol concentrations (1–8 g/L). Results suggested that the affinity (K_m) of glucose-grown strains for glycerol (2.2 g/L for GDHE, 0.9 g/L for GAT and GAN) were significantly higher than those of cells harvested from sorbitol (2.6–3.0 g/L). In addition, a 15% increase in apparent V_{max} was obtained for glucose-grown strains compared to those cultivated on sorbitol. Table 4 also indicated that for three adaptively evolved strains, a 30–40% increase in overall catalytic activity (0–2 h) was observed when glucose-grown cells were used as biocatalysts instead of sorbitol-grown cells.

When the substrate concentration was increased to 40 g/L, the overall catalytic rate decreased slightly compared to the case where 10 g/L glycerol was used. In general, it took less than 24 h for glucose-grown GAT and GAN cells to complete the biotransformation reactions, whereas it was much longer for GAT and GAN cells harvested from sorbitol medium. In addition, the glucose-grown GAT and GAN cells converted all glycerol to DHA, while about 5–10 g/L of residual glycerol was left in the reactions where sorbitol-grown GAT or GAN cells were used. In comparison with the nearly complete conversion of glycerol to DHA at low glycerol concentration (Fig. 3c), the catalytic properties of most cells had deteriorated at 40 g/L of glycerol (especially the sorbitol-grown cells), which might be partly attributed to the inactivation of some enzymes and the induction of glyceric acid formation under high glycerol and DHA conditions (Bories et al., 1991; Claret et al., 1992; Habe et al., 2010).

Significantly enhanced catalytic performances of both GAT and GAN cells were observed with 40 g/L of glycerol in comparison with that of the GDHE strain (Fig. 3b and d), which was, however, not apparent in the oxidation of 10 g/L of glycerol (Fig. 3a and c). This indicates that the knockout of the *adh* gene and overexpression of the *sldAB* gene are advantageous to the biotransformation of high concentration of glycerol. Moreover, in accord with the catalytic activity data in Table 4, the glucose-grown GAT or GAN cells were more advantageous to the oxidation of glycerol to DHA than the cells obtained from the sorbitol medium. However, much remains unknown about why the sorbitol-cultivated cells exhibited less effective catalytic performance and how glucose medium influences the activity of key enzymes in this biotransformation system.

When supplied with 40 g/L glycerol, the DHA yield of glucose-grown GAN strain was up to 99.2% (the maximum catalytic rates reached to approximately 4.7 g/gCDW/h), increased by 86% compared to that of sorbitol-grown GDHE (53.2%). Our unpublished data indicated that when glycerol concentration was increased to 100 g/L glycerol in Erlenmeyer flasks, a DHA yield of 88.1 g/L was obtained by glucose-grown GAN strain. In addition, oxygen supply is one of the key factors limiting the biotransformation of glycerol to DHA by using strains cultivated in flask-scale fermentation, and the DHA production capability of glucose-grown strains could be much enhanced in the transformation process with higher rotation speed (data not show). Moreover, the DHA production could also be markedly improved by using specific bioreactor systems where a threshold value of DHA could be set to prevent the inhibition of biotransformation caused by high DHA concentration (Hekmat et al., 2003).

3.4. Quantitative real-time PCR (qRT-PCR) analysis of sldAB gene

In order to investigate the effect of mSLDH expression on the biotransformation of glycerol, quantitative real-time PCR analysis was conducted for *G. oxydans* mutants grown on sorbitol (80 g/L) or glucose (20 g/L) to late-exponential phase. The obtained mSLDH expression data were normalized to the expression level in sorbi-

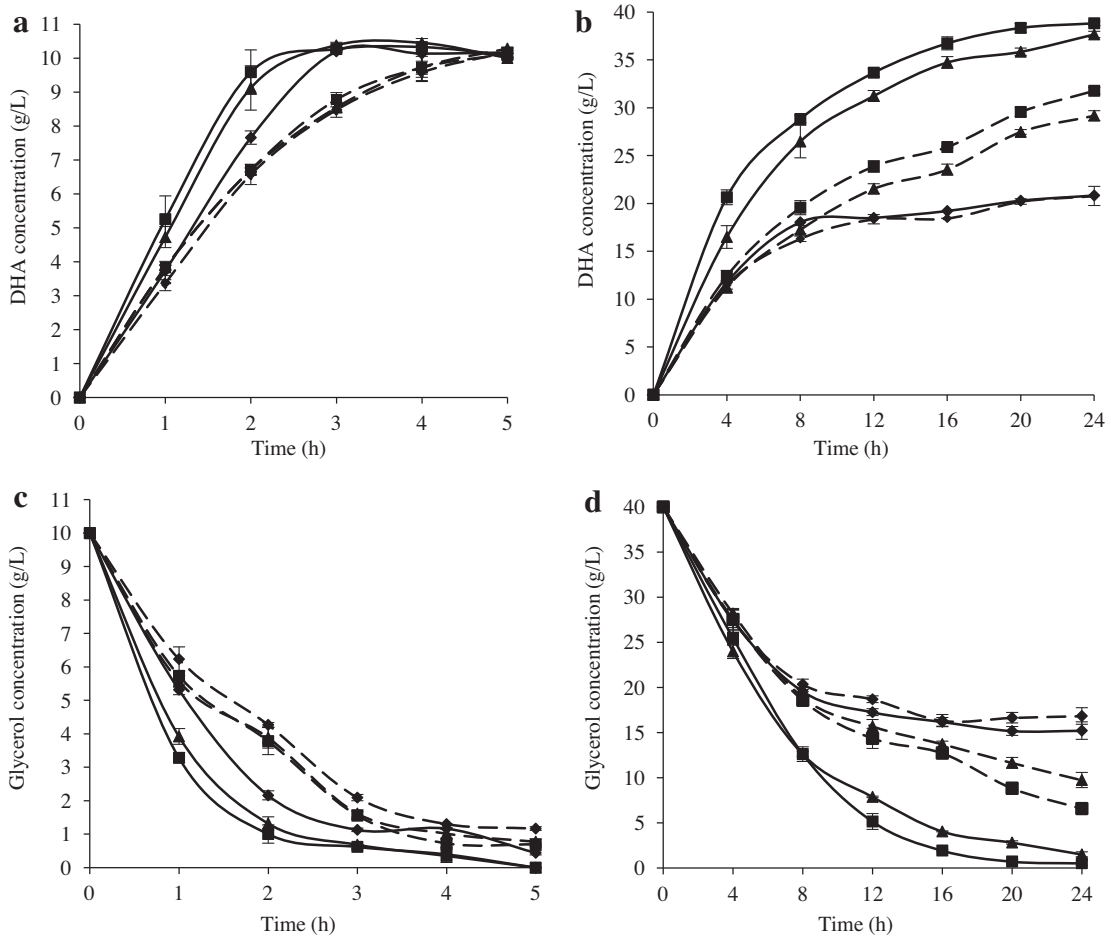

Fig. 3. Comparison of DHA production and glycerol consumption among GDHE (diamonds), GAT (triangles) and GAN (squares) cultivated sorbitol (dashed line) or glucose medium (solid line). (a) Biotransformation of 10 g/L glycerol to DHA. (b) Biotransformation of 40 g/L glycerol to DHA. (c) Consumption of 10 g/L glycerol. (d) Consumption of 40 g/L glycerol.

Table 4
Comparison of overall catalytic rates (0–2 h) by different resting cells harvested from sorbitol or glucose medium.

g/gCDW/h	Glycerol 10 g/L		Glycerol 40 g/L	
Strains	Sorbitol medium	Glucose medium	Sorbitol medium	Glucose medium
GDHE	3.16 ± 0.01	4.37 ± 0.02	3.12 ± 0.02	4.06 ± 0.01
GAT	3.43 ± 0.01	4.86 ± 0.03	3.35 ± 0.04	4.62 ± 0.01
GAN	3.48 ± 0.01	4.87 ± 0.03	3.39 ± 0.02	4.67 ± 0.01

tol-grown GDHE cells and shown in Fig. 4. The most significant results were that the mSLDH expression levels of all glucose-grown mutants were about 10-fold greater than those cells cultivated in sorbitol medium, indicating that the transcription of the *sldAB* gene in *G. oxydans* was markedly induced by glucose. It was also found that due to the overexpression of the *sldAB* gene in GAT or GAN strains, a two- to threefold increase in transcription was verified for these strains compared with that of the GDHE strain. In addition, qRT-PCR analysis indicated that the native *sldAB* promoter in the GAN cells presented a slightly higher transcriptional efficiency than the *tufB* promoter, which was, however, not in agreement with the result obtained by Gatgens et al. (2007).

3.5. Effects of glucose medium on biotransformation of glycerol to DHA

The studies showed that the GAN strain grown on glucose (20 g/L) exhibited best biotransformation performance. This strain was then selected to investigate how the glucose medium affects the cell growth and the biotransformation of glycerol to DHA. Cultures with various glucose concentrations (0.5%, 1%, 2%, 3%, 4%) were carried out and it was found that the maximum cell density (C_{max}) did not increase significantly with the increase of glucose concentration. Approximately 0.7 gDCW/L of the GAN biomass was obtained in the culture with 0.5% glucose, whereas higher glucose concentrations resulted in only slight improvement in cell growth (approximately 1.0 gDCW/L obtained on 1–4% glucose).

Toyama et al. (2005) found that the membrane-bound pyrroloquinoline quinone (PQQ)-dependent sorbitol dehydrogenase in a thermotolerant *Gluconobacter frateurii* strain could not be induced by D-sorbitol, and the efficiency of DHA production from glycerol was kept unvaried under different concentrations of sorbitol. In comparison, in case that glucose concentration was below 3%, the conversion of glycerol to DHA in the early biotransformation phase was enhanced with the increase of glucose concentration

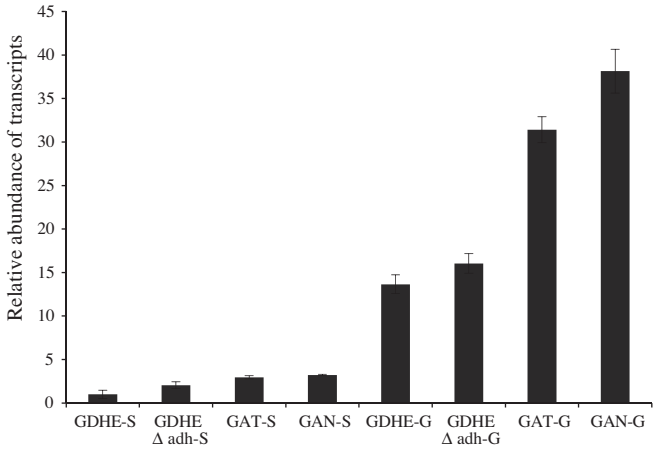

Fig. 4. Comparison of mSLDH expression in different *G. oxydans* strains cultivated on 80 g/L of sorbitol (S) or 20 g/L of glucose (G). All cells grown to late-exponential phase were harvested for total RNA isolation.

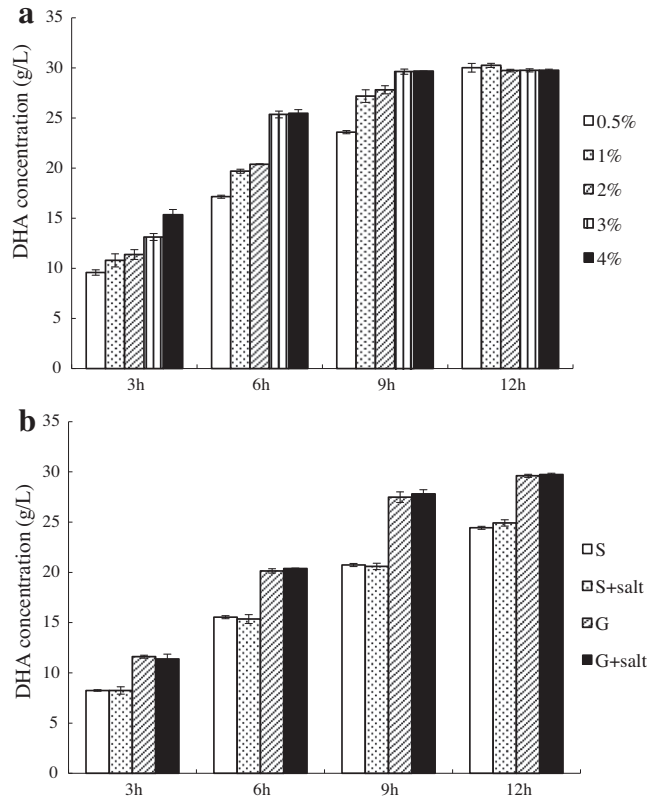

Fig. 5. Comparison the effect of medium component to catalytic properties of GAN. (a) The effect of saline solution (salt) in sorbitol medium (S) or glucose medium (G) to catalytic properties. (b) The effect of different concentration of glucose in glucose medium to catalytic properties.

(Fig. 5a), indicating the activity of mSLDH might be induced by a higher concentration of glucose. The effects of saline solution on the biotransformation were also studied with the GAN cells grown in the medium with or without the addition of KH_2PO_4 1.5 g/L, $(NH_4)2SO_4$ 1.5 g/L, and $MgSO_4{\cdot}7H_2O$ 0.5 g/L. No changes in catalytic activity were observed for the GAN cells cultivated under different saline conditions (Fig. 5b), suggesting that the standard saline solution had less effects on the biotransformation performance of the *G. oxydans* mutants constructed in this study.

It was the first study that focused on the systematic construction of glucose-metabolizing *G. oxydans* strains to lower the production cost for DHA biotransformation from glycerol. Experimental results showed significant increases in sldAB transcription in glucose-grown strains compared to those in cells cultivated on sorbitol, which therefore resulted in markedly enhanced DHA biotransformation from glycerol (Figs. 3 and 4). Glucose is therefore supposed to be an effective inducer for mSLDH. It is known that the mSLDH in *G. oxydans* is an enzyme with broad substrate specificity including sorbitol, arabitol, and D-gluconate, although the affinity to individual substrates might vary (Adachi et al., 2001; Matsushita et al., 2003). Further and definite proof is required to show if glucose-grown strains constructed in this study are capable of converting a variety of substrates to industrially important chemicals, and inducing the expression of other membrane-bound enzymes.

4. Conclusion

The purposes of using glucose as the sole carbon source for the constructed *G. oxydans* strains were not only to reduce the production cost by obtaining biomass more efficiently, but also to enhance the catalytic properties in industrial biotransformation processes of DHA. The results obtained in this study indicated a preliminary achievement of these purposes and a possibility of further optimization of the strain and biotransformation systems for better DHA production.

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