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Q6 Original Research Article

Metabolic engineering of *Corynebacterium glutamicum* for the de novo production of ethylene glycol from glucose

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ABSTRACT

Development of sustainable biological process for the production of bulk chemicals from renewable feedstock is an important goal of white biotechnology. Ethylene glycol (EG) is a large-volume commodity chemical with an annual production of over 20 million tons, and it is currently produced exclusively by petrochemical route. Herein, we report a novel biosynthetic route to produce EG from glucose by the extension of serine synthesis pathway of *Corynebacterium glutamicum*. The EG synthesis is achieved by the reduction of glycoaldehyde derived from serine. The transformation of serine to glycoaldehyde is catalyzed either by the sequential enzymatic deamination and decarboxylation or by the enzymatic decarboxylation and oxidation. We screened the corresponding enzymes and optimized the production strain by combinatorial optimization and metabolic engineering. The best engineered *C. glutamicum* strain is able to accumulate 3.5 g/L of EG with the yield of 0.25 mol/mol glucose in batch cultivation. This study lays the basis for developing an efficient biological process for EG production.

1. Introduction

With the growing concern over environmental crisis and fossil-energy depletion, development of sustainable biological process using renewable bioresources to produce energy, materials and chemicals is becoming an appealing approach for chemical industry. The fast development of analytical and engineering tools from metabolic engineering, systems and synthetic biology in the past two decades has substantially accelerated the process of strain engineering to produce both natural and non-natural products (Chen et al., 2010, 2015; Chen and Zeng, 2013). Biological production of bulk chemicals at commercial scale, such as butanol (Lan and Liao, 2013), 1,3-propanediol (Nakamura and Whited, 2003) and 1,4-butanediol (Yim et al., 2011), has been achieved by intensive strain modifications integrating different rational engineering tools. Herein, we report the production of ethylene glycol (EG), another important large-volume commodity chemical, from renewable feedstock (e.g. glucose) by de novo pathway design and metabolic engineering.

EG is a very important platform chemical which can be used as raw material for polymers, anti-freezing agent and coolant etc. The primary application of EG is to synthesize over 25 million tons of polyethylene terephthalate (PET) annually. Currently, EG is mainly produced based on chemical route using ethylene derived from the petrochemical industry. Although ethylene could now be produced by chemical dehydration of bioethanol (Zhang and Yu, 2013), a more economical route to manufacture EG directly from bio-feedstock is highly desirable. Development of low-cost fermentation process to produce EG from carbohydrate is an important alternative towards such goal. To the best of our knowledge, no natural microorganism can produce EG from glucose. Only marginal accumulation of EG as a metabolic byproduct was previously identified during the degradation of arabinose by some microorganism such as *Caldicellulosiruptor saccharolyticus* (Isern et al., 2013). Recently, the biosynthesis routes of EG from pentoses have been clarified and metabolic engineering of *Esherichia coli* has enabled the direct fermentation of arabinose or xylose to produce EG (Liu et al., 2013; Stephanopoulos et al., 2013). However, the theoretical yield of the proposed pathways is only 1 mol EG/mol pentose (0.4 C-mol/C-mol), making this approach economically unfavorable. Furthermore, other abundant sugars, such as sucrose and glucose, cannot be utilized as substrates based on the proposed pentose degradation pathway. The need for a more efficient process led us to evaluate other potential pathways for EG production.

In this study, we proposed that EG production can be achieved by the extension of serine synthesis pathway as illustrated in Fig. 1A (see detailed description in the Results session). Serine is a natural amino acid that can be synthesized by most of microorganisms. High accumulation of serine has been previously demonstrated by metabolic engineering of *Corynebacterium glutamicum* or *E. coli* (Gu

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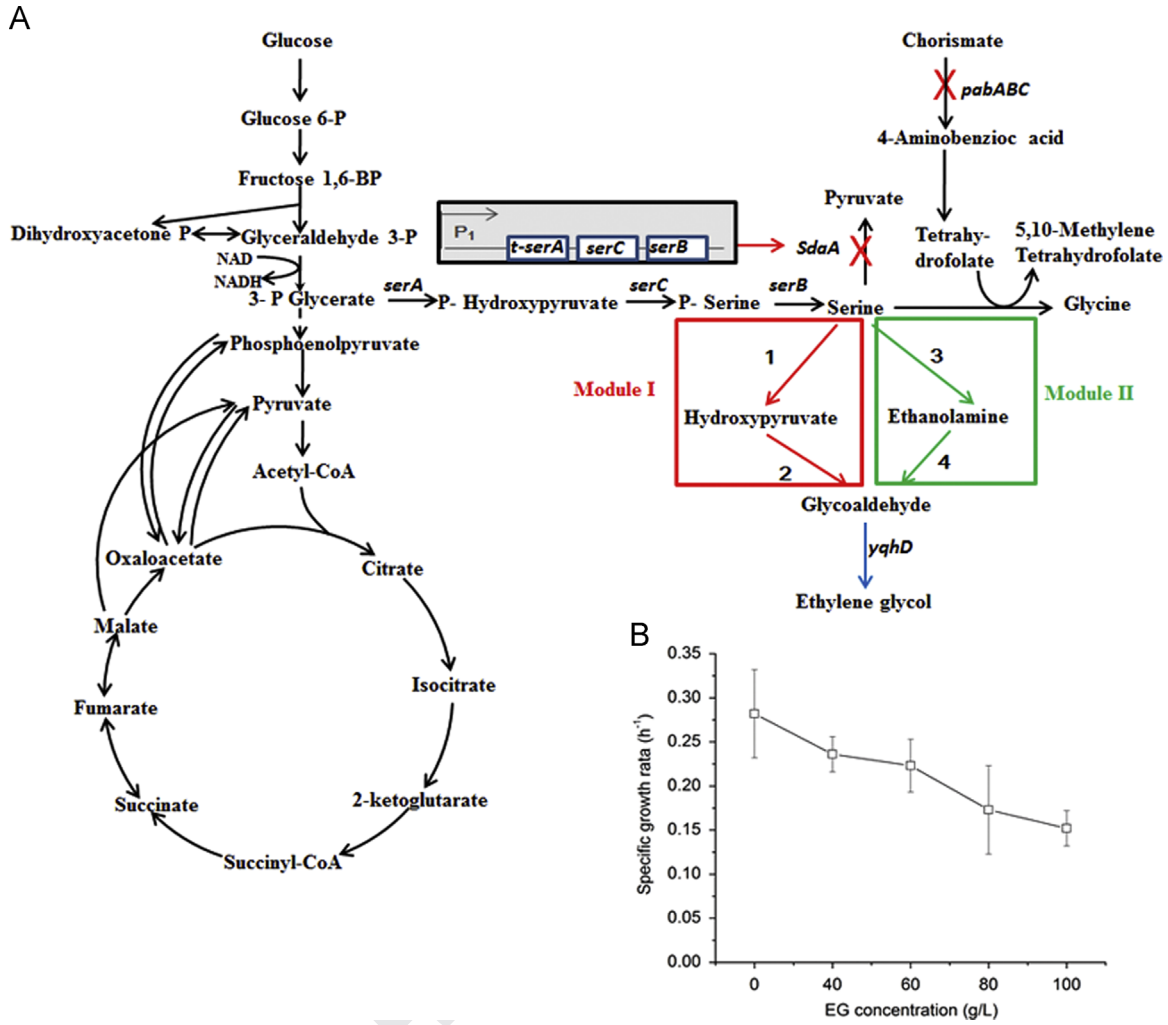

Fig. 1. Proposed routes for ethylene glycol (EG) production derived from serine synthesis pathway (A) and effect of EG for the cell growth of *Corynebacterium glutamicum* (B). (A) Serine can be transferred into glycoaldehyde via two synthetic modules: (1) deamination of serine to hydroxypyruvate by aminotransaminase or amino acid dehydrogenase, and decarboxylation of hydroxypyruvate to glycoaldehyde by α-ketoacid decarboxylase (module I); (2) decarboxylation of serine to ethanolamine by serine decarboxylase, and oxidation of ethanolamine to glycoaldehyde by monoamine oxidase (module II). Glycoaldehyde can be reduced to EG by alcohol dehydrogenase such as *yqhD*. To enhance the precursor supply for EG synthesis, the *pabABC* operon encoding the aminodeoxychorismate synthase and aminodeoxychorismate lyase was deleted. *SdaA* gene encoding serine deaminase was substituted by an artificial *serACB* operon, containing truncated *serA* (with a deletion of 197 amino acids at the C terminus), *serC* and *serB* under the control of a strong constitutive promoter. (B) To evaluate the toxicity of EG, *C. glutamicum* was cultured in LB medium with 25 g/L glucose and different concentrations of EG at 30 °C and 200 rpm.

et al., 2014; Stolz et al., 2007; Zhu et al., 2014). *C. glutamicum* is a gram-positive bacterium which can utilize various substrates for the production of different amino acids in industry (Bommareddy et al., 2014; Chen, et al., 2014; Hasegawa et al., 2013). Recently, *C. glutamicum* has also been engineered to produce other bulk chemicals such as isobutanol, cadaverine, succinate, etc. (Blombach et al., 2011; Buschke et al., 2011; Litsanov et al., 2012). In this study, we selected a prophage-free *C. glutamicum* strain MB001 (Baumgart et al., 2013) as the chassis to reconstruct the EG synthesis route based on the extension of its natural serine synthesis pathway. By systematical enzyme screening and combinatorial pathway assembling, we demonstrated that it was possible to directly produce EG from glucose by metabolically engineered *C. glutamicum*.

2. Materials and methods

2.1. Bacterial strains and plasmids

Strains and plasmids used in this study are listed in Table 1. *E. coli* DH5αMCR (Invitrogen) was used for the construction of pK18mob*sacB* derived suicide vector for gene deletion and substitution in *C. glutamicum* (Schäfer et al., 1994). *E. coli* DH5α was routinely used for the construction of other plasmids. *C. glutamicum* MB001 is a prophage-free strain derived from wildtype *C. glutamicum* ATCC13032 (Baumgart et al., 2013). Gibson assembly cloning kit (NEB) was used to construct all of the plasmids (Gibson et al., 2009).

2.2. Construction of suicide vectors and C. glutamicum mutants

Gene knockout or substitution in *C. glutamicum* MB001 was achieved by a two-step homologous recombination process based on the suicide vector pK18mob*sacB* as described before (Chen et al., 2011). All of the constructed strains were verified by colony PCR using appropriate primers.

To delete *pabABC* operon encoding the aminodeoxychorismate synthase and aminodeoxychorismate lyase (nucleotides 1,054,917–1,057,051), pK18mob*sacB-pabABC* was constructed. Primers pabABC-up-F and pabABC-up-R were used to amplify a 1000-bp fragment of the upstream of *pabABC* and primers pabABC-down-F and pabABC-down-R was used to amplify a 1000-bp fragment of the downstream

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Table 1
Strains and plasmids used for this study.

Strain or plasmid	Description	Source or reference
E. coli Strains		
DH5α	Host for plasmid construction	TIANGEN BIOTECH
DH5αMCR	Host for suicide plasmid construction	Invitrogen
C. glutamicum Strains		
MB001	Derived from ATCC 13032, prohage-free	Baumgart et al. (2013)
PAB1	MB001 with in-frame deletion of *pabABC*	This study
PABS1	PAB1 with in-frame substitution of *sdaA* with an artificial operon *serACB*	This study
Plasmids		
pK18mob*sacB*	Suicide vector	Schäfer et al. (1994)
pK18mob*sacB- pabABC*	Vector enabling deletion of *pabABC*	This study
pK18mob*sacB- serACB*	Vector enabling substitution of *sdaA* with *serACB*	This study
pEC-K18mob2	*E. coli/C. glutamicum* shuttle vector, Km^r, containing pGA1 ori	Tauch et al. (2002)
pCRB1	*E. coli/C. glutamicum* shuttle vector, Cm^r, containing pBL1 ori	Nataka et al. (2004)
pEC-P1-yqhD	pEC-K18mob2 containing *E. coli yqhD* gene under the control of a constitutive promoter P1	This study
pCRB-P1-yqhD	pCRB1 containing *E. coli yqhD* under the control of promoter P1	This study
pEC-P1-LlKivD-yqhD	pEC-K18mob2 containing *L. lactis kivD* and *E. coli yqhD* under the control of promoter P1	This study
pEC-P1-PpMdlc-yqhD	pEC-K18mob2 containing *P. putida mdlC* and *E. coli yqhD* under the control of promoter P1	This study
pEC-P1-MtSucA-yqhD	pEC-K18mob2 containing *M. tuberculosis sucA* and *E. coli yqhD* under the control of promoter P1	This study
pEC-P1-ScPdc-yqhD	pEC-K18mob2 containing *S. cerevisiae pdc5* and *E. coli yqhD* under the control of promoter P1	This study
pEC-P1-TaPdc-yqhD	pEC-K18mob2 containing *T. aestivum pdc* and *E. coli yqhD* under the control of promoter P1	This study
pEC-P1-AgAGT-PpMdlc-yqhD	pEC-K18mob2 containing *A. thaliana sgt, P. putida mdlC* and *E. coli yqhD* under the control of promoter P1	This study
pEC-P1-TmSAT-PpMdlc-yqhD	pEC-K18mob2 containing *T. maritima sat, P. putida mdlC* and *E. coli yqhD* under the control of promoter P1	This study
pEC-P1-RnSPT-PpMdlc-yqhD	pEC-K18mob2 containing *R. norvegicus spt, P. putida mdlC* and *E. coli yqhD* under the control of promoter P1	This study
pEC-P1-ScVdh-PpMdlc-yqhD	pEC-K18mob2 containing *S. cinnamonensis vdh, P. putida mdlC* and *E. coli yqhD* under the control of promoter P1	This study
pEC-P1-TiPdh-PpMdlc-yqhD	pEC-K18mob2 containing *T. intermedius pdh, P. putida mdlC* and *E. coli yqhD* under the control of promoter P1	This study
pEC-P1-GsLdh-PpMdlc-yqhD	pEC-K18mob2 containing *G. stearothermophilus ldh, P. putida mdlC* and *E. coli yqhD* under the control of promoter P1	This study
pCRB-P1-ASAO-yqhD	pCRB1 containing *Arthrobacter sp.* AO and *E. coli yqhD* under the control of promoter P1	This study
pCRB-P1-EcMaoA-yqhD	pCRB1 containing *E. coli maoA* and *E. coli yqhD* under the control of promoter P1	This study
pCRB-P1-AtSdc-ASAO-yqhD	pCRB1 containing *A. thaliana sdc, Arthrobacter sp. AO* and *E. coli yqhD* under the control of promoter P1	This study
pCRB-P1-OsSdc-ASAO-yqhD	pCRB1 containing *O. sativa sdc, Arthrobacter sp. AO* and *E. coli yqhD* under the control of promoter P1	This study

of pabABC operon. The resulting PCR fragments were inserted into the EcoRI restriction site of pK18mob*sacB* by Gibson assembly.

Plasmid pK18mob*sacB-serACB* was made to replace *sdaA* gene (encoding serine deaminase) with an artificial operon *serACB*. The artificial serine synthesis operon *serACB* consists of three genes (truncated *serA* encoding the 333 N-terminal amino acids of phosphoglycerate dehydrogenase, *serC* and *serB* encoding phosphoserine transaminase and phosphoserine phosphatase) under the control of a strong promoter P1(5′-TTGACATTAATTTGAATCTGTGTTATAATGGTTC-3′) (Rytter et al., 2014). The PCR primers were used to amplify the corresponding fragments including the upstream and downstream homologous arms of *sda* gene, the truncated *serA*, *serC* and *serB*. All of the fragments were assembled into the EcoRI restriction site of pK18*mobsacB* by Gibson assembly as illustrated in Supplementary Fig. 1. The primers used in this session are listed in Supplementary Table 1.

2.3. Plasmid construction for EG synthesis modules

The *yqhD* gene of *E. coli* with a strong synthetic promoter P1 (5′-TTGACATTAATTTGAATCTGTGTTATAATGGTTC-3′) was amplified by using the primers of yqhD-F and yqhD-R. The fragment was inserted into the EcoRI restriction site of *E. coli*/*C. glutamicum* shuttle vector pEC-K18mob2 and pCRB1, resulting in the construction of plasmids pEC-P1-yqhD and pCRB-P1-yqhD, respectively. The codon-optimized gene sequences of the candidate enzymes for EG synthesis module 1 and 2 (Figs. 2 and 3) were synthesized (Qinglan Biotechnology, China). All of the synthesized genes contain the same prefix sequence (5′-AATTGGAAACTTTTT*AGAAAG*GTGTGTTG-3′) and suffix sequence (5′-AGAGACGGAGTCACTGCCAA-3′) in order to introduce an identical RBS spacer and to facilitate the following gene assembly. The Uniprot accession numbers of the candidate enzymes are listed in Supplementary Table 2. The synthesized genes were inserted into plasmid pEC-P1-yqhD (module I) or pCRB-P1-yqhD (module II) by the standard protocol of Gibson assembly. The primers used in this session are listed in Supplementary Table 3.

2.4. Culture condition and analytical method

The modified CGXII minimal medium was used for the characterization of *C. glutamicum* mutants (Keilhauer et al., 1993), consisting of (per liter): 40 g glucose, 20 g $(NH_4)_2SO_4$, 5 g urea, 0.25 g $MgSO_4 \cdot 7H_2O$, 1 g KH_2PO_4, 1 g K_2HPO_4, 42 g 3-morpholinopropanesulfonic acid, 10 mg $CaCl_2$, 10 mg $FeSO_4 \cdot 7H_2O$, 10 mg $MnSO_4 \cdot H_2O$, 1 mg $ZnSO_4 \cdot 7H_2O$, 0.2 mg $CuSO_4$, 0.02 mg $NiCl_2 \cdot 6H_2O$, 0.2 mg biotin, and 30 mg protocatechuic acid. Shake-flask fermentation was performed in 500 ml baffled flasks containing 50 ml CGXII minimal medium. The cell was grown at 30 °C and 150 rpm agitation. When appropriate, the medium was supplemented with 10 μg/ml chloramphenicol and/or 25 μg/ml kanamycin.

For the large-scale quantification, batch fermentations were carried out in 5 L bioreactors with the working volume of 2 L. The medium consists of (per liter) 40 g glucose, 30 g $(NH_4)_2SO_4$, 2 g corn steep liquor, 3 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 30 mg protocatechuic acid, 20 mg $FeSO_4 \cdot 7H_2O$, 20 mg$MnSO_4 \cdot H_2O$, 0.5 mg biotin, 450 μg thiamine, 10 mg chloramphenicol and 25 mg kanamycin. The fermentations were maintained at 30 °C, pH 7.2 (controlled by feeding NH_4OH), and aeration of 1 vvm. Dissolved oxygen was controlled at 30% saturation by adjusting the agitation speed.

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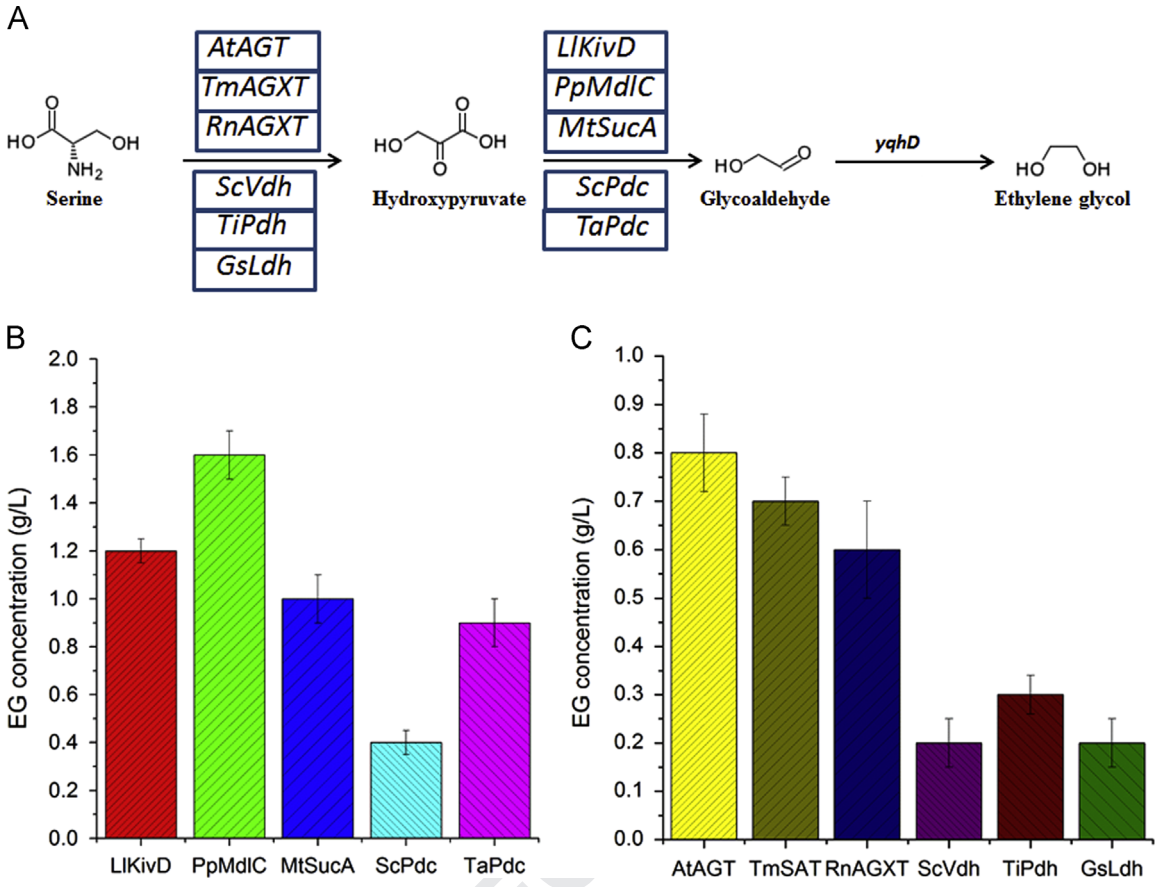

Fig. 2. EG synthesis by module I. (A) metabolic pathway of synthetic module I. The candidate enzymes tested in this study are illustrated. (B) EG production by the strains with different decarboxylases. *E. coli yqhD* was expressed in all of the strains. The cells were cultured in shake flasks with CGXII minimal medium with the addition of 10 g/L hydroxypyruvate. Data were taken from 72 h of cultivation. (C) EG production by the strains with different aminotransaminases or amino acid dehydrogeanses. *P. putida mdlC* and *E. coli yqhD* were expressed in all of the strains. The cells were cultured in shake flasks with CGXII minimal medium with the addition of 10 g/L serine. Data were taken from 72 h of cultivation.

Quantification of glucose, EG and other organic acids were carried out by using High performance liquid chromatography (HPLC) equipped with a Aminex HPX-87H Column (300×7.8 mm^2) using 0.005 M H_2SO_4 as the mobile phase with a flow rate of 0.6 mL/min, and detection via refractive index or UV absorption at 210 nm (Bommareddy et al., 2014). The extracellular amino acids were quantified by HPLC after derivatizing with 6-Aminoquinolyl-N-Hydroxysuccinimidyl carbamate (Chen et al., 2014). Cell concentration was determined at an optical density of 660 nm and by dry biomass measurements.

3. Results and discussion

3.1. Designing novel EG synthetic routes based on the extension of serine synthesis pathway

To the best of our knowledge, there is no natural pathway that can directly convert glucose or other hexoses to EG. In this study, we proposed two artificial routes for EG production based on the extension of serine synthesis pathway (Fig. 1). Both routes start from serine and converge at glycoaldehyde. The first route (module I) is activated by the deamination of serine to hydroxypyruvate (reaction 1). This step can be realized by aminotransferase or amino acid dehydrogenase. Transformation of hydroxypyruvate to glycoaldehyde can be catalyzed by α-ketoacid decarboxylase (reaction 2). The second route (module II) also consists of two steps: serine is firstly converted into ethanolamine by serine decarboxylation (reaction 3); and ethanolamine is then oxidized to glycoaldehyde by monoamine oxidase (reaction 4). Glycoaldehyde can be reduced to EG by alcohol dehydrogenase.

In most of microorganisms, serine shares a common synthesis pathway derived from glycolysis intermediate 3-phosphate glycerate by three enzymatic steps (Fig. 1A). This pathway has been engineered for serine production in *C. glutamicum* or *E. coli* (Gu et al., 2014; Stolz et al., 2007; Zhu et al., 2014). *C. glutamicum* has been proved to be a superior host for serine production. Moreover, *C. glutamicum* can tolerate high concentration of EG (Fig. 1B). Thus, we decided to engineer the proposed pathways into *C. glutamicum*. Before the experiment, we evaluated the theoretical maximum yield of the new pathways. The theoretical yield for the proposed pathways is 2 mol EG per mol glucose (0.6 C-mol/C-mol). This is much higher than that of pentose-derived pathways which give only 0.4 C-mol/C-mol (Liu et al., 2013; Stephanopoulos et al., 2013). It should also be noticed that the maximum mass yield of EG (0.69 g/g glucose) is much higher than ethanol (0.51 g/g glucose). Currently, the price of EG is about USD 1000–1200/ton which is higher than ethanol (http://www.sunsirs.com/). Considering that the downstream process of EG is more complicated than ethanol, the titer and yield of EG should be higher than 100 g/L and 0.5 g/g sugar in order to make the biological process to be economically competitive to chemical route. Thus, metabolic engineering of industrial strain to efficiently utilize cheap sugars such as lignocellulose with high titer and yield is highly important for the biological production of EG.

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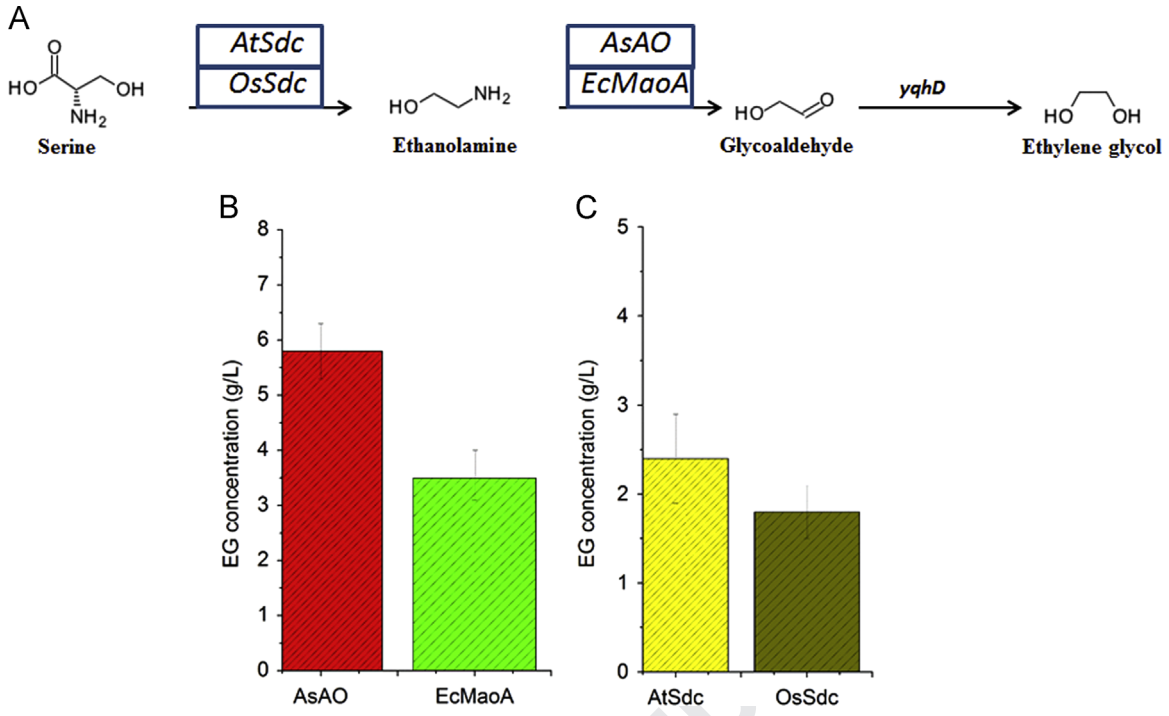

Fig. 3. EG synthesis by module II. (A) metabolic pathway of synthetic module II. The candidate enzymes tested in this study are illustrated. (B) EG production by the strains with different monoamine oxidase. *E. coli yqhD* was expressed in all of the strains. The cells were cultured in shake flasks with CGXII minimal medium with the addition of 10 g/L ethanolamine. Data were taken from 72 h of cultivation. (C) EG production by the strains with different serine decarboxylase. *Arthrobacter sp.* amine oxidase (AO) and *E. coli yqhD* were expressed in all of the strains. The cells were cultured in shake flasks with CGXII minimal medium with the addition of 10 g/L serine. Data were taken from 72 h of cultivation.

Most of the reactions in the proposed pathways lack the corresponding specific enzymes. Thus, the key challenge is to screen the efficient enzymes. The NADH-dependent lactaldehyde oxidoreductase (encoded by *fucO*) and NADPH-dependent non-specific alcohol dehydrogenase (encoded by *yqhD*) have been reported to be able to catalyze transformation of glycoaldehyde to EG (Lee et al., 2010; Liu et al., 2013; Obradors et al., 1998). The NADPH-dependent YqhD has been widely used for the production of various alcohols, e.g. 1, 3-propanediol. It was shown to be move favorable for aldehyde reduction than NADH-dependent alcohol dehydrogenase (Nakamura and Whited, 2003). Thus, YqhD was selected for the catalysis of the last artificial step. The *E. coli yqhD* gene was PCR amplified and cloned into *E. coli/C. glutamicum* shuttle vector pEC-K18mob2 or pCRB1 under the control of a strong constitutive promoter P1 (Rytter et al., 2014). The resulting plasmids were designated as pEC-P1-yqhD and pCRB-P1-yqhD, respectively. The *yqhD* gene can be highly expressed in *C. glutamicum* MB001 with both vectors, giving the specific activities of 21 U/mg and 24 U/mg towards glycoaldehyde reduction. We then focused our attention to search for prospective enzymes for module I and II.

3.2. Conversion of serine to EG by module I

Module I includes two reactions: the deamination of serine to hydroxypyruvate, and the decarboxylation of hydroxypyruvate to glycoaldehyde (Fig. 2). So far, no corresponding enzymes are known for the specific catalysis of these two reactions. We then explore enzyme promiscuity to fill the gaps in order to complete the whole pathway. The deamination of serine can be catalyzed by aminotransferase (E.C. 2.6.1-) or amino acid dehydrogenase (E.C. 1.4.1-). The decarboxylation of hydroxypyruvate can be catalyzed by α-ketoacid decarboxylase (E.C. 4.1.1-).

For the second reaction, the activity of hydroxypyruvate decarboxylase (EC 4.1.1.40) has been previously reported in mammalian systems (Hedrick and Sallach, 1964) and wheat germ (Davies and Asker, 1984). However, the activity is not associated with any gene (or amino acid sequence) to date. It was supposed that the decarboxylation of pyruvate and hydroxypyruvate in wheat germ was catalyzed by the same enzyme. We then carried out a BLASTP of wheat genome (*Triticum aestivum*) to search for the potential enzyme using the amino acid sequence of pyruvate decarboxylase of *Saccharomyces cerevisiae* (ScPdc, uniprot accession number P16467). An uncharacterized protein (uniprot accession number A0A077RT78) was identified which showed 34% of sequence identity with ScPdc. This protein is designated as TaPdc. We also selected several other promiscuous α-ketoacid decarboxylase, including α-ketoisovalerate decarboxylase from *Lactococcus lactis* (LlKivD) (de la Plaza et al., 2004), benzoylformate decarboxylase from *Pseudomonas putida* (PpMdlC) (Niu et al., 2003), α-ketoglutarate decarboxylase from *Mycobacterium tuberculosis* (MtSucA) (Tian et al., 2005). We synthesized all of the selected decarboxylases with optimized codons and coexpressed them with YqhD in *C. glutamicum* MB001. When cultured in CGXII minimal medium with the addition of 10 g/L hydroxypyruvate, all of the constructed recombinants can produce EG (Fig. 2B). No EG was accumulated for the control strain which only expressed YqhD. The strain harboring TaPdc can produce about 0.9 g/L EG, indicating that TaPdc is able to catalyze the decarboxylation of hydroxypyruvate. Specifically, PpMdlc and LlKivD were shown to be more efficient than other decarboxylases in our experiments. These two enzymes were also able to catalyze the decarboxylation of 5-hydroxy-α-ketoglutarate for 1, 4-butanediol production (Liu and Lu, 2015). We then select PpMdlC to continue the following screening.

The activity of serine transamination has been identified in several plants (Liepman and Olsen, 2001; Zhang et al., 2013) and a few bacteria (Yang et al., 2008). This type of enzyme, called as serine–glyoxylate aminotransferase or alanine–glyoxylate aminotransferase (AGT), can use different types of amino acid donors and organic acid acceptors for the transamination. We selected

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Table 2
EG production by *C. glutamicum* mutants.

C. glutamicum strain	EG concentration [g/L]
PABS1	ND
PABS1(pEC-P1-AgAGT-PpMdlc-yqhD)	0.7 ± 0.1
PABS1 (pCRB-P1-AtSdc-ASAO-yqhD)	1.7 ± 0.3
PABS1 (pEC-P1-AgAGT-PpMdlc-yqhD, pCRB-P1-AtSdc-ASAO-yqhD)	2.2 ± 0.2

ND: not detectable.

three serine aminotransferases, including AGT from *Arabidopsis thaliana* (AtAGT) (Liepman and Olsen, 2001), AGT from *Thermotoga maritima* (TmSAT) (Yang et al., 2008), and AGT from *Rattus norvegicus* (RnAGXT), to examine their capacities to catalyze serine transamination in *C. glutamicum*. Moreover, we also tried to screen several amino acid dehydrogenases which have been previously shown to be able to use a broad scope of substrates (Fig. 2A). These enzymes are valine dehydrogenase from *Streptomyces cinnamonensis* (ScVdh) (Turnbull et al., 1997), phenylalanine dehydrogenase from *Thermoactinomyces intermedius* (TiPdh) (Kataoka and Tanizawa, 2003), leucine dehydrogenase from *Geobacillus stearothermophilus* (GsLdh) (Kataoka and Tanizawa, 2003). We synthesized all of the selected enzymes with optimized codons and coexpressed them with PpMdlC and YqhD in *C. glutamicum* MB001. When cultured in CGXII minimal medium with the addition of 10 g/L serine, all of the constructed recombinants can produce EG (Fig. 2C). No EG was accumulated for the control strain which only expressed PpMdlC and YqhD. The strain harboring AtAGT showed the highest efficiency in our experiments which can produce about 0.8 g/L EG. Thus, we have successfully filled the gap of module I for EG production.

3.3. Conversion of serine to EG by module II

The conversion of serine to glycoaldehyde can also be catalyzed by serine decarboxylase (E.C. 4.1.1.-) and ethanolamine oxidase (E.C. 1.4.3.8) (Fig. 3A). Serine decarboxylase has been found in some plants (Rontein et al., 2001). The activity of ethanolamine oxidase was detected in *Pseudomonas sp.* while the sequence is still unknown (Narrod and Jakoby, 1964). Alternatively, the oxidation of ethanolamine can be catalyzed by non-specific monoamine oxidase. For example, *E. coli* monoamine oxidase (EcMaoA) was reported to be able to use a broad scope of substrates (Roh et al., 1994). Ota et al. (2008) also reported an amine oxidase from *Arthrobacter sp.* (ASAO) that can utilize ethanolamine as substrate. We tested these two enzymes by overexpression of the synthesized genes together with YqhD in *C. glutamicum* MB001. Both enzymes can utilize ethanolamine as substrate for EG synthesis while ASAO was more efficient (Fig. 3B).

To identify the efficient serine decarboxylase (Sdc), we examined the Sdc from *A. Thaliana* (ATSdc) and Sdc from *Oryza sativa* (OsSdc). By overexpression ATSdc or OsSdc together with ASAO and YqhD in *C. glutamicum* MB001, the mutant can produce 2.3 g/L or 1.8 g/L EG in CGXII minimal medium with 10 g/L serine (Fig. 3C). It is proved that production of EG through module II is also feasible.

3.4. Metabolic engineering of C. glutamicum for the direct conversion of glucose to EG

To engineer *C. glutamicum* for the direct production of EG from glucose, it is necessary to enhance the availability of serine for EG synthesis. In *C. glutamicum*, the serine synthesis pathway as well as its degradation pathways are quite active. Serine can be quickly degraded to pyruvate by serine dehydratase (encoded by *sdaA* gene) or to glycine by serine hydroxymethyltransferase (encoded by *glyA* gene) (Fig. 1). The *glyA* gene is essential for the growth of *C. glutamicum* and thus cannot be knocked out. Since tetrahydrofolate (THF) is essential for the activity of *glyA*, we tried to limit the supply of THF by deleting the *pabABC* operon encoding aminodeoxychorismate synthase and aminodeoxychorismate lyase. *PabABC* knockout blocks p-aminobenzioc acid (PABA) synthesis, making the strain (PAB1) to be THF auxotrophic. Limiting the feeding of PABA or THF can be used to control the activity of *glyA*. A similar strategy has been previously used to enhance serine production (Stolz et al., 2007). To further direct the flux to serine synthesis, we in-frame substituted the *sda* gene with an artificial *serACB* operon. The artificial *serACB* operon consists of a truncated *serA* encoding feedback-insensitive phosphoglycerate dehydrogenase (Peters-Wendisch et al., 2002), *serC* and *serB* under the control of a strong constitutive promoter P1 (Rytter et al., 2014). The resulting strain, designated as PABS1, can accumulate about 9.0 g/L serine in CGXII minimal medium with the addition of 0.1 mM folate.

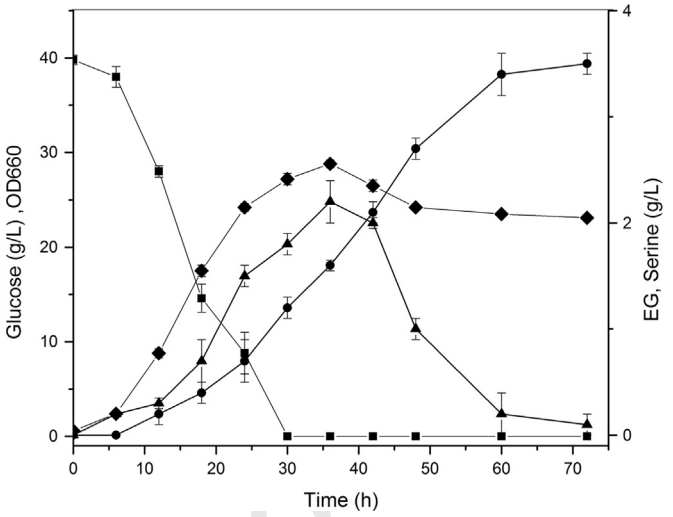

Fig. 4. Profiles of cell growth (◆), glucose (■), EG (●) and serine (▲) concentrations during the batch fermentation by *C. glutamicum* strain PABS1 (pEC-P1-AgAGT-PpMdlc-yqhD, pCRB-P1-AtSdc-ASAO-yqhD) on complex medium.

We then introduced the previously constructed EG synthesis pathways into *C. glutamicum* PABS1 and tested EG production with CGXII minimal medium (Table 2). The strain containing synthesis module I, PABS1 (pEC-P1-AgAGT-PpMdlc-yqhD), accumulated 0.7 g/L EG in shake flask. The strain containing synthesis module II, PABS1 (pCRB-P1-AtSdc-ASAO-yqhD), accumulated 1.7 g/L EG. The strain containing both plasmids, PABS1 (pEC-P1-AgAGT-PpMdlc-yqhD, pCRB-P1-AtSdc-ASAO-yqhD), produced 2.2 g/L EG.

To further evaluate the performance of PABS1 (pEC-P1-AgAGT-PpMdlc-yqhD, pCRB-P1-AtSdc-ASAO-yqhD), batch fermentation was carried out in 5 L bioreactor with complex medium. As shown in Fig. 4, the strain started to produce EG after 6 h and accumulated a final concentration of 3.5 g/L at 72 h. The yield of EG is 0.25 mol/mol glucose. Serine was initially accumulated and then consumed when glucose was depleted which was mainly transferred to EG.

4. Conclusion

In this study, we proposed two routes for EG production by the extension of serine synthesis pathway. The proposed pathways can be generally used for converting different substrates to EG with the theoretical yield of 2 mol EG/mol sugar. By screening the potential enzymes, we have filled the gaps between the proposed

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non-natural pathways and the available natural enzymes. By further engineering the serine synthesis pathway, we are able to construct *C. glutamicum* strains that can directly utilize glucose for EG production. The present study lays the basis for developing an efficient biological process for EG production.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ymben.2015.10.013.

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