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ORIGINAL PAPER

# Recombinant expression of *glpK* and *glpD* genes improves the accumulation of shikimic acid in *E. coli* grown on glycerol

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**Abstract** Shikimic acid (SA) is an industrially important chiral compound used in diverse commercial applications, and the insufficient supply by isolation from plants and expensive chemical synthesis of SA has increased the importance of developing strategies for SA synthesis. In our previous studies, glycerol was observed to be an effective carbon source for SA accumulation in *E. coli* DHPYAAS-T7, where the PTS operon (*ptsHIcrr*) and *aroL* and *aroK* genes were inactivated, and the *tktA*, *glk*, *aroE*, *aroF*$^{fbr}$, and *aroB* genes were overexpressed. For further investigation of the effects of glycerol aerobic fermentation on SA accumulation in *E. coli* BL21(DE3), the *glpD*, *glpK* genes and *tktA*, *glk*, *aroE*, *aroF*$^{fbr}$, *aroB* genes were overexpressed simultaneously. The results indicated that SA production was increased 5.6-fold, while the yield was increased 5.3-fold over that of parental strain in shake flasks. It is demonstrated that the aerobic fermentation of glycerol associated with *glpD* and *glpK* gene overexpression increased glycerol flux, resulting in higher SA accumulation in *E. coli* BL21(DE3)-P-DK.

**Keywords** Shikimic acid · Aerobic fermentation · Glycerol · *GlpD* gene · *GlpK* gene

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## Introduction

Shikimic acid (SA) is an important chiral compound used as a key chemical building block for the antiviral drug Oseltamivir (Tamiflu) (Johansson and Lidén 2006; Kancharla et al. 2009). SA is an intermediate of the aromatic amino acid biosynthesis pathway found in microbes and plants (Adachi et al. 2006). In recent years, SA has been used in the formulation of many industrially important chemical products, such as indole, aromatic amino acids and alkaloids (Bochkov et al. 2012). Isolation in insufficient quantities from plants and the high demand for SA during the pandemic of swine flu has increased the importance of this compound. Thus, fermentation using microorganisms gained our attention for large-scale production. Compared with extraction from plants, microbial fermentation has a variety of advantages, and *E. coli* is commonly applied in large-scale fermentation and industrial production. In order to improve shikimic acid's availability, fermentative production processes from renewable resources like glucose and glycerol present an excellent and even more sustainable alternative to meet the current market volume at a competitive price level.

Microbial production of shikimic acid involves the shikimic acid pathway as shown in Fig. 1. Genetic manipulations and metabolic engineering strategies have been applied to *E. coli* for shikimic acid accumulation, and the four primary approaches are listed as follows. Firstly, the shikimic acid pathway begins with phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P), which can be enhanced via metabolic engineering of the glycolytic and pentose phosphate pathway, respectively. Overexpression of the *tktA* gene (coding for transketolase) results in the increase of shikimic acid yield from 38 to 52 g/l, due to enhancing the concentration of E4P (Knop et al. 2001). Inactivation of the PTS

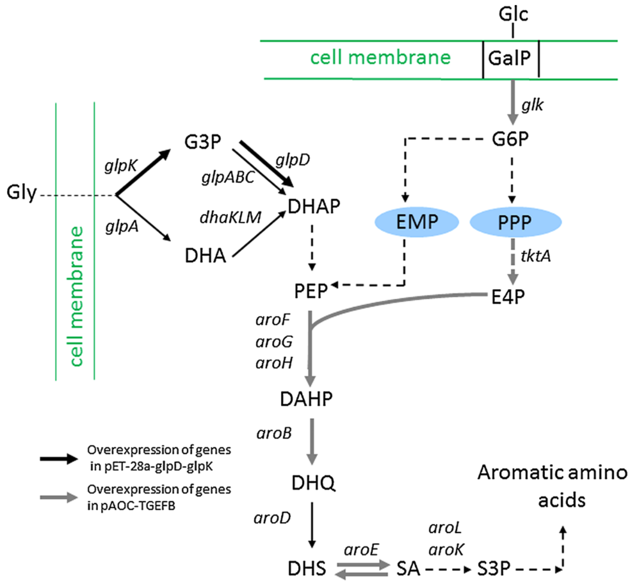

**Fig. 1** Pathways involved in glycerol dissimilation and biosynthesis of shikimic acid in *E. coli* BL21(DE3). Abbreviations: G3P, glycerol-3-phosphate; DHA, dihydroxyacetone; DHAP, dihydroxyacetone phosphate; PEP, phosphoenol pyruvate; DAHP, 3-deoxy-D-arabinoheptulosonate-7-P; DHQ, 3-dehydroquinic acid; DHS, 3-dehydroshikimic acid; SA, shikimic acid; E4P, erythrose-4-P; S3P, shikimate-3-phosphate; PPP, pentose phosphate pathway; EMP, glycolytic pathway; G6P, glucose-6-P; GlaP, galactose permease; Glc, glucose. Relevant reactions are represented by the names of the gene(s) coding for the enzymes: *glpK,* glycerol kinase; *glpD*, aerobic glycerol-3-phosphate dehydrogenase; *glpABC*, anaerobic glycerol-3-phosphate dehydrogenase; *glpA*, glycerol dehydrogenase; *dhaKLM*, dihydroxyacetone kinase; *tktA*, transketolase I; *glk*, glucokinase; *aroF, aroG, aroH,* DAHP synthase isoenzymes F, G and H, respectively; *aroB*, DHQ synthase; *aroD*, DHQ dehydratase; *aroE*, shikimic dehydrogenase; *aroK*, shikimate kinase I; *aroL*, shikimate kinase II. *Broken lines* or *arrows* represent two or more enzymatic reactions or incompletely characterized reactions. *Continuous arrows* represent unique reactions catalysed by one or more enzymes

operon (PTS$^-$), expression of non-PTS glucose transporters genes such as *glf* (coding for glucose facilitators) and *glk* (coding for glucokinase) in combination with overexpression of *tktA* gene are reported to increase the production of shikimic acid to 71 g/l (Chandran et al. 2003; Gibson et al. 2001). Secondly, overexpression or integration of the genes for the key enzymes (e.g. *aroH*, *aroF* and *aroG* coding for DAHP (3-deoxy-D-arabinoheptulosonate-7-phosphate) synthase isoenzymes, *aroB* coding for dehydroquinate synthase, *aroE* coding for shikimate dehydrogenase) involved in the three rate-limiting step of shikimic acid pathway can increase its production. The expression of a six-gene group ($aroG^{fbr}$, *aroB*, *aroD*, *aroE*, *tktA* and *zwf*), under the control of a synthetic operon constructed in a laboratory-evolved strain bearing simultaneous PTS$^-$ and pykF$^-$, has been recently reported to increase the shikimic acid yield on glucose to 42 % mol/mol, which represents the highest reported yield (Rodriguez et al. 2013). Thirdly, preventing shikimic acid transformation to aromatic amino acids by blocking or down-regulating the downstream pathway after the SA production stage is an effective way. Deleting the *aroK* and *aroL* genes (coding for shikimate kinase I and II) can block aromatic amino acid pathway flux from central carbon metabolism. For example, in *E. coli* DHPYAAS-T7, where the PTS operon (*ptsHIcrr*), *aroL* and *aroK* genes are inactivated, and the *tktA*, *glk*, *aroE*, $aroF^{fbr}$ and *aroB* genes were overexpressed, the shikimic acid production increased to 1,850 mg/l (Chen et al. 2012). Finally, reducing the by-products from the three reversible reactions catalysed by shikimic acid dehydrogenase, DHQ dehydratase and quinic acid dehydrogenase accumulated in SA synthesis. Overexpression of feedback-resistant (fbr) DAHP synthase genes (e.g. $aroF^{fbr}$ and $aroG^{fbr}$) can avoid the feedback inhibition in DADH transforming (Shinada et al. 1998; Rodriguez et al. 2013).

In this work, we first investigated the effects of glycerol aerobic fermentation associated with the genes coding for glycerol-3-phosphate dehydrogenase (*glpD*) and glycerol kinase (*glpK*) on SA accumulation. The plasmid pET-28a-glpD–glpK was constructed to increase glycerol metabolic flux. Three engineered *E. coli* strains were constructed: *E. coli* BL21(DE3)-P-DK, in which the *glpD* and *glpK* genes of the glycerol aerobic metabolic pathway and the *tktA*, *glk*, *aroE*, $aroF^{fbr}$ and *aroB* genes of the aromatic amino acid pathway were overexpressed, *E. coli* BL21(DE3)-DK, in which the *glpD* and *glpK* genes were overexpressed and *E. coli* BL21(DE3)-P, in which the *tktA*, *glk*, *aroE*, $aroF^{fbr}$ and *aroB* genes were overexpressed. The data showed that glycerol aerobic fermentation associated with *glpD* and *glpK* genes overexpression increased glycerol metabolic flux, resulting in higher SA accumulation in *E. coli* BL21(DE3)-P-DK (5.6-fold more than *E. coli* BL21), suggesting that glycerol metabolic flux in *E. coli* BL21(DE3)-P-DK was very important for SA accumulation and glycerol.

## Materials and methods

### Strains, plasmids and genetic methods

T4 DNA ligase, restriction enzymes and *Taq* DNA polymerase were purchased from TaKaRa Biotechnology Co., Ltd. (Dalian, China). DNA molecular weight markers were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). The *glpD* gene was amplified from *E. coli* genomic DNA using primers Pf-glpD/Pr-glpD. The PCR products were purified and cloned into the cloning vector pMD19-T. The DNA fragment digested by *Sac*I/*Hin*dIII was ligated into the corresponding restriction sites of pET-28a to construct pET-28a-glpD. The ribosomal binding site (RBS AAGAAGGAG) and spacer (sequence between RBS and

**Table 1** Strains, plasmids and primers

| Strains and plasmids | Relevant characteristics[a] | |
|---|---|---|
| *E. coli* BL21(DE3) | Wild type, Expression host | This work |
| *E. coli* BL21(DE3)-DK | *E. coli* BL21(DE3) derivative, harboring pET-28a-glpD–glpK | This work |
| *E. coli* BL21(DE3)-P | *E. coli* BL21(DE3) derivative, harboring pAOC-TGEFB | This work |
| *E. coli* BL21(DE3)-P-DK | *E. coli* BL21(DE3) derivative, harboring pET-28a-glpD–glpK and pAOC-TGEFB | This work |
| *E. coli* DH5α-T7 | *E. coli* DH5α derivative, *ΔptsHIcrr ΔaroLΔaroK*, Knock-in of T7-RNA-pol gene | Chen et al. (2012) |
| *E. coli* DH5α-T7-DK | *E. coli* DH5α-T7 derivative, harboring pET-28a-glpD–glpK | This work |
| *E. coli* DH5α-T7-P | *E. coli* DH5α-T7 derivative, harboring pAOC-TGEFB | This work |
| *E. coli* DH5α-T7-P-DK | *E. coli* DH5α-T7 derivative, harboring pET-28a-glpD–glpK and pAOC-TGEFB | This work |
| *Plasmids* | | |
| pAOC | LacO P15Aori, $Cm^r$ | Chen et al. (2012) |
| pMD19-T | LacZ, $Amp^r$ | This work |
| pET-28a | LacI, $Kan^r$ | This work |
| pAOC-TGEFB | 6.0-kb *Bgl*II-*Eco*RI fragment containing *aroE*, *aroB*, site-specific mutagenesis *glk, tktA, aroF*$^{fbr}$ gene of *E. coli* cloned into pAOC, $Cm^r$ | Chen et al. (2012) |
| pET-28a-glpD–glpK | 3.0-kb *Xho*I-*Sac*I fragment containing *glpK* and *glpD* gene of *E. coli* into pET-28a, $Kan^r$ | This work |
| *Primers* | | |
| Pf-glpD | 5′ CGAGCTCATGGAAACCAAAGATCTGATTG 3′ | This work |
| Pr-glpD | 5′ CCCAAGCTTTTACGACGCCAGCGATAACCTCTGC 3′ | This work |
| Pf-glpK | 5′ CCCAAGCTT*AAGAAGGAG*ATATACCATGACTGAAAAAAAATATATCGTTG 3′ | This work |
| Pr-glpK | 5′ CCGCTCGAGACATTATTCGTCGTGTTCTTCC 3′ | This work |
| RTf-glpD | 5′ CCACCAGCCACTGACTCACA 3′ | This work |
| RTr-glpD | 5′ CATTACGCTCGCACTTACGG 3′ | This work |
| RTf-glpK | 5′ GTTGGAAGGTGCGGTGTTTA 3′ | This work |
| RTr-glpK | 5′ GTGCAGACGGATACCAGAGT 3′ | This work |
| RTf-16s | 5′ TTCCAGACTCCTACGGGAC 3′ | This work |
| RTr-16s | 5′ TATTACCGCGGCTGCTGGC 3′ | This work |

[a] $Kan^r$, $Cm^r$, and $Amp^r$ indicate kanamycin, chloramphenicol and ampicillin resistance, respectively

the starting codon, ATATACC) from the pET-28a vector were used to ligate *glpD* and *glpK*. The *glpK* fragment with the RBS and spacer sequence was amplified using primers Pf-glpK/Pr-glpK and *E. coli* genomic DNA. The PCR products were purified and inserted into the vector pMD19-T. After digested with *Hin*dIII/*Xho*I, the DNA fragment was cloned into the corresponding restriction sites of pET-28-glpD to construct pET-28a-glpD–glpK. PCR reactions were carried out in total 50 µl reaction solution, containing 5 U of *Taq* DNA polymerase, 20 ng *E. coli* genomic DNA, 1.0 µM of each primer, and 250 µM dNTP. The amplifications were performed for 30 cycles: 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1.5 min, and plus an additional 10 min at 72 °C after the cycles. Oligonucleotide primers were synthesized by Genescript Co., Ltd. (Nanjing, Jiangsu, China). All resulting plasmids were sequenced by Genescript Co., Ltd. (Nanjing, Jiangsu, China). The genetically stable strains were stored in 25 % glycerol at −80 °C. Plates were prepared using LB medium containing 2 % agar, kanamycin (50 µg/ml) and chloramphenicol (25 µg/ml).

*E. coli* BL21(DE3) and *E. coli* DH5α-T7 were used as expression hosts. The pET-28a was used as expression vectors. The plasmid pAOC-TGEFB was kindly provided by Dr. Fang (Zou et al. 2004). *E. coli* BL21(DE3)-P and *E. coli* BL21(DE3)-DK were constructed by transforming plasmid pAOC-TGEFB and pET-28a-glpD–glpK, respectively, into the parental strain *E. coli* BL21(DE3). The plasmid pAOC-TGEFB and pET-28a-glpD–glpK were transformed into *E. coli* BL21(DE3) to form *E. coli* BL21(DE3)-P-DK. *E. coli* DH5α-T7-P and *E. coli* DH5α-T7-DK were obtained by transforming plasmid pAOC-TGEFB and pET-28a-glpD–glpK, respectively, into *E. coli* DH5α-T7. The plasmid pAOC-TGEFB and pET-28a-glpD–glpK were transformed into *E. coli* DH5α-T7 to form *E. coli* DH5α-T7-P-DK. *E. coli* strains were transformed by the calcium chloride methods. All strains, along with the primers and plasmids used in this study are listed in Table 1.

### Cultivation conditions

Prior to fermentations, the strains of interest were streaked from their frozen glycerol stocks onto appropriate LB plates consisting of 5 g/l yeast extract, 10 g/l peptone and 5 g/l NaCl and incubated overnight at 37 °C. Three colonies were used to inoculate into 2 ml of LB medium supplemented (when needed) with 25 μg/ml chloramphenicol and 50 μg/ml kanamycin. The culture was incubated overnight at 37 °C. 1 ml of this culture was transferred into 50 ml of modified M9 medium consisting of (/l) 7 g $K_2HPO_4{\cdot}3H_2O$, 3 g $NaH_2PO_4{\cdot}3H_2O$, 2.5 g·NaCl, 0.5 g $MgSO_4{\cdot}7H_2O$, 10 g peptone, 15 g yeast extract and 20 g glycerol. The filled volume was 50 ml in 250-ml erlenmeyer flask with agitation at 200 rev/min and the modified M9 medium was adjusted to pH 7.8 with 1 M NaOH. IPTG was used with a final concentration of 1 mM to induce the overexpression of genes in the *E. coli* BL21(DE3) derivative strains and L-arabinose (L-ara) was used with a final concentration of 3 g/l to induce the overexpression of genes in the *E. coli* DH5α-T7 derivative strains. All of the fermentation experiments were performed in triplicate. The data was averaged and presented as the mean standard deviation, and statistical significance was defined as $P < 0.05$.

### Analysis of gene amplification by RT-PCR

RNA extraction from *E. coli* was performed using Bacterial Total RNA Isolation Kit (Sangon Biotech Co., Ltd, Shanghai, China). First-strand cDNA synthesis was according to Su et al. (2012). *glpD* was amplified using primers RTf-glpD/RTr-glpD, *glpK* was amplified using primers RTf-glpK-RTr-glpK, and the internal control 16S rDNA was amplified using primers RTf-16s/RTr-16s. The amplification was performed for 30 cycles: 94 °C for 30 s, 51 °C for 30 s, and 72 °C for 30 s; plus an additional 10 min at 72 °C after the cycles. 2 % (w/v) agarose gel electrophoresis was performed to analyse PCR product and Bio-Imaging System with the Quantity One 1-D analysis software (Bio-Rad, CA, USA) was used to analyse the band intensity.

### Glycerol-3-phosphate dehydrogenase and glycerol kinase activity assay

The glycerol-3-phosphate dehydrogenase was assayed as described previously (Peres et al. 2010). Briefly, the reaction mixture was prepared as follows: 2.6 mM $NAD^+$, 11 mM glycerol-phosphate, 2.5 mM $MgCl_2{\cdot}6H_2O$ and 50 mM glycine/NaOH buffer at pH 9.5. The reaction was started by adding cell lysate to the assay mixture with the final reaction volume of 3 ml and ended by adding 2 % SDS after incubating at 25 °C for 3 min. 2 % SDS was added before adding cell lysate in blank control. NADH formation was detected at 340 nm. One enzymatic unit was defined as the amount of enzyme required to form 1 μM of NADH (molar absorption coefficient of 6,200 $M^{-1}$ $cm^{-1}$) per min at 25 °C under the conditions specified. The glycerol kinase activity was assayed as described previously (Hayashi and Lin 1967). The phosphorylation of glycerol was coupled to the reduction of $NAD^+$ by the action of L-α-glycerol-P dehydrogenase in glycine/NaOH buffer at pH 9.5. The reaction mixture contained the following: 10 mM glycerol, 5 mM ATP, 2.5 mM $MgCl_2{\cdot}6H_2O$, 3 mM $NAD^+$, 500 μM sodium carbonate, 20 U of L-α-glycerol-P dehydrogenase, and cell lysate in a final volume of 3.0 ml. The L-α-glycerol-P dehydrogenase was omitted from the blank. The UV detection wavelength was 340 nm as above. The unit of enzyme activity was expressed as 1 μM of L-α-glycerol-P formed per min at 25 °C under the conditions specified. Protein concentration was determined by the method of Lowry with bovine serum albumin as a standard.

### SDS-PAGE

Protein molecular weight markers were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). The supernatant from the cell lysate harboring expression proteins was used for SDS-PAGE. The expression of recombinant plasmids was detected by SDS-PAGE. The concentration of resolving gel and stacking gel were 12 and 5 %, respectively. Protein concentration was determined by the same method mentioned above.

### Analytical methods

Biomass concentrations were determined every few hours until the end of the culture period. Samples (2 ml) were collected, and the dry cell weight (DCW) was monitored. For the determination of SA accumulation in the fermentation broth, samples were prepared by centrifugation at 4,500*g* for 3 min (TGL-16G, Anting Scientific Instrument Factory, Shanghai, China), and the supernatant was filtered through 0.45 μm nylon membranes. Glycerol concentration and HPLC analysis were carried out according to Chen et al. (2012). HPLC was performed with Shimadzu LC-10A instrument with a SPD-20A UV–visible diode-array detector. The mobile phase was deionized water adjusted to pH 2.0 with $H_3PO_4$. Filtered samples of SA were chromatographed on a reverse-phase Hedera ODS-2 column (4 × 150 mm; 5 μm; Hanbon Science and Technology, Huaian, Jiangsu, China) and the flow rate was 0.8 ml $min^{-1}$ at 30 °C. The detection wavelength was 217 nm.

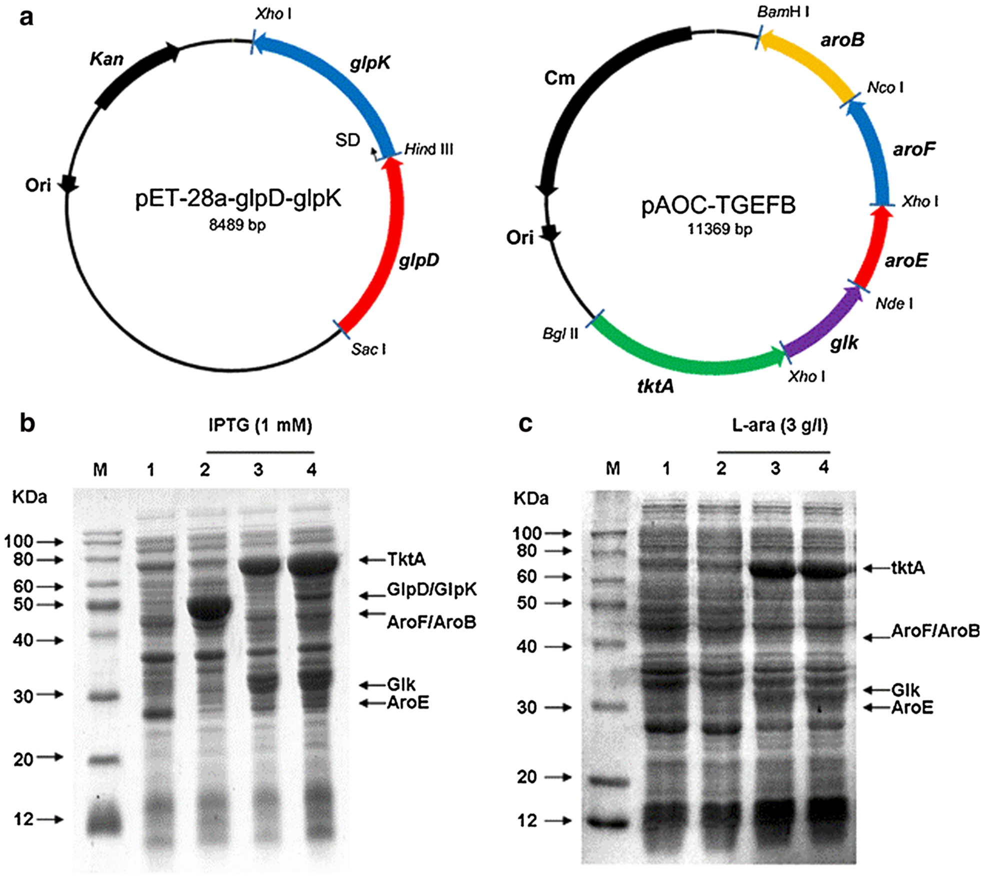

**Fig. 2** The construction of pET-28a-glpD–glpK and expression of enzymes. **a** The maps of polycistronic plasmids. **b** Overexpression of the vector pET-28a-glpD–glpK and pAOC-TGEFB in *E. coli* BL21(DE3) derivative strains. The lane names are denoted as follows: M, protein marker; 1, *E. coli* BL21(DE3) wild type; 2, *E. coli* BL21(DE3)-DK; 3, *E. coli* BL21(DE3)-P; 4, *E. coli* BL21(DE3)-P-DK. **c** Overexpression of the vector pET-28a-glpD–glpK and pAOC-TGEFB in *E. coli* DH5α-T7 derivative strains. The lane names are denoted as follows: M, protein marker; 1, *E. coli* DH5α-T7; 2, *E. coli* DH5α-T7-DK; 3, *E. coli* DH5α-T7-P; 4, *E. coli* DH5α-T7-P-DK; (TktA, 72 kDa; GlpK/GlpD, 55 kDa; AroF/AroB, 43 kDa; Glk, 35 kDa; AroE, 29 kDa)

## Results

### Construction of pET-28a-glpD–glpK and overexpression of *glpK* and *glpD* genes

To investigate the effects of glycerol aerobic fermentation associated with *glpK* and *glpD* genes on SA accumulation, *glpK* and *glpD* genes were cloned into pET-28a under the control of the T7 promoter, while five genes of the SA pathway, containing *tktA*, *glk*, *aroE*, *aroF*$^{fbr}$ and *aroB*, were cloned into the plasmid pAOC-TGEFB (provided by Dr. Fang, Zou et al. 2004) (Fig. 2a). As shown in Fig. 2b, 55 kDa GlpD/GlpK were notably overexpressed (lane 2), the same as 72 kDa TktA, 43 kDa AroF/AroB, 35 kDa Glk and 29 kDa AroE (lane 3). Seven proteins, GlpD, GlpK, TktA, AroF, AroB, Glk and AroE, were observed as distinct bands (lane 4), indicating that plasmids pET-28a-glpD–glpK and pAOC-TGEFB could be compatible and expressed in *E. coli* BL21(DE3)-P-DK. As shown in Fig. 2c, the *glpD* and *glpK* genes were not expressed in *E. coli* DH5α-T7-DK and *E. coli* DH5α-T7-P-DK (lanes 2 and 4), suggesting that the expression of pET-28a-glpD–glpK in the PTS$^{-}$ system background of *E. coli* DH5α-T7 was not well regulated. To further investigate the transcriptional level of *glpK* and *glpD* genes, SqRT-PCR was performed among the strains as above (Fig. 3a–d). As shown in Fig. 3c, d, the transcription ratio of *glpD* in *E. coli* BL21 (DE3)-P-DK was 2.3-fold than *E. coli* BL21(DE3), while the ratios in *E. coli* DH5α-T7-DK and *E. coli* DH5α-T7-P-DK were no obvious differences with *E. coli* BL21(DE3). In *E. coli* BL21(DE3)-P-DK, the transcription ratio of *glpK* was 2.0-fold higher than *E. coli* BL21(DE3), and the ratios in *E. coli* DH5α-T7-DK and *E. coli* DH5α-T7-P-DK showed no obvious differences with *E. coli* BL21(DE3). To further investigate translational level of *glpD* and *glpK*, the activities of glycerol-3-phosphate dehydrogenase and glycerol kinase were detected (Fig. 3e, f). The activities of glycerol-3-phosphate dehydrogenase in *E. coli* BL21 (DE3), *E. coli* BL21 (DE3)-P-DK, *E. coli* DH5α-T7-DK and *E. coli* DH5α-T7-P-DK were 0.023, 0.057, 0.025, and 0.024 U/mg, respectively. The activities of glycerol kinase in the strains above were 0.019, 0.045, 0.018, and 0.02 U/mg, respectively. It was shown that the enzyme activity in *E. coli* BL21(DE3)-P-DK was obviously higher than that of the other three strains. The date of RT-PCR and enzyme activity indicated that *glpD* and *glpK* genes were co-expressed effectively in *E. coli* BL21(DE3), but not in *E. coli* DH5α-T7-DK or

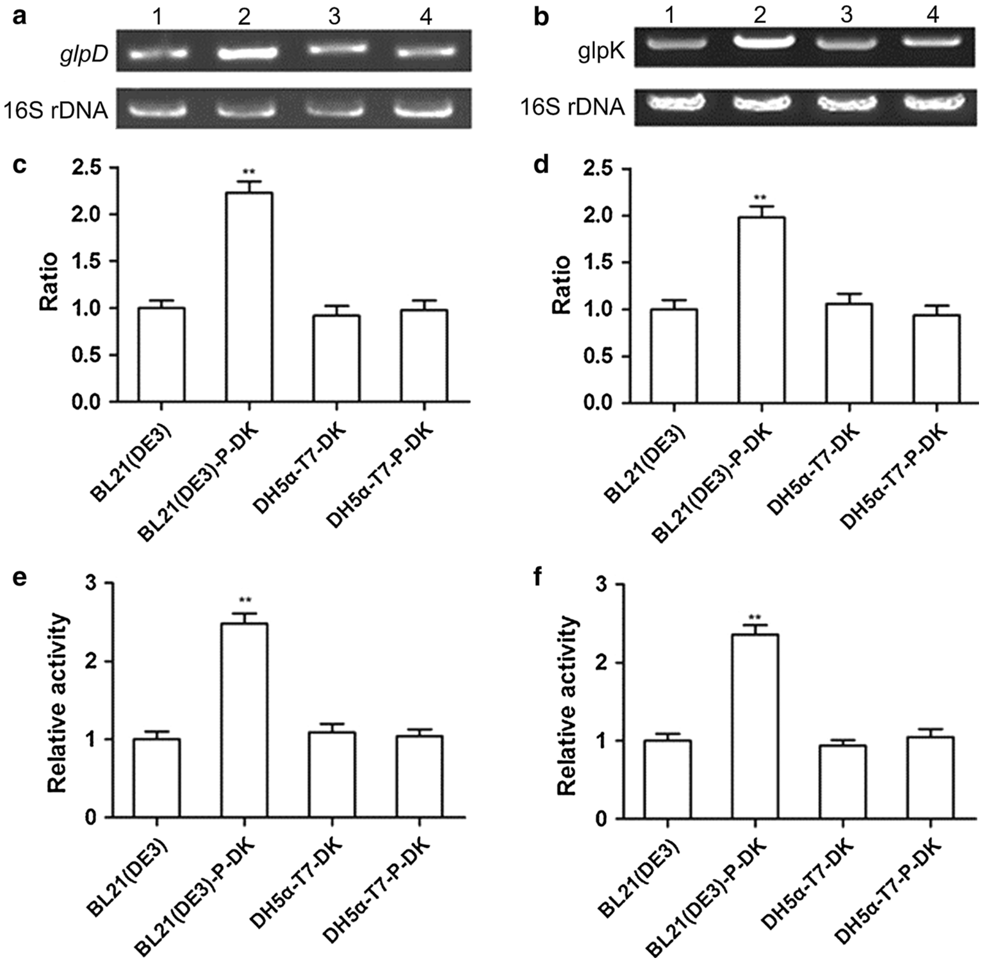

**Fig. 3** RT-PCR analysis of *glpD* and *glpK* genes and the enzyme activity assay of glycerol-3-phosphate dehydrogenase and glycerol kinase. **a** RT-PCR products of *glpD* and16S rDNA, **b** RT-PCR products of *glpK* and16S rDNA, **c** the ratios of *glpD* transcription level in the recombinant groups to that of the *E. coli* BL21(DE3) wild type, **d** the ratios of *glpK* transcription level in the recombinant groups to that of the *E. coli* BL21(DE3), **e** the relative activity of glycerol-3-phosphate dehydrogenase compared to *E. coli* BL21(DE3), **f** the relative activity of glycerol kinase compared to *E. coli* BL21(DE3). Housekeeping gene 16S rDNA was an internal reference. *Lanes 1*, *2*, *3* and *4* stood for BL21(DE3), Bl21(DE3)-P-DK, DH5α-T7-DK and DH5α-T7-P-DK respectively. The ratios of *glpD*, *glpK* transcription level in the recombinant group compared to that of the wild type group were calculated after the BL21 (DE3) normalized to 1. *$P < 0.05$, **$P < 0.01$ and compared with BL21 (DE3) group

*E. coli* DH5α-T7-P-DK. Due to the apparent poor expression of *glpD* and *glpK*, we had to change from past host *E. coli* DH5α-T7 to a BL21 (DE3), three engineered *E. coli* strains were constructed: *E. coli* BL21(DE3)-DK, in which the *glpD* and *glpK* genes of the glycerol aerobic metabolic pathway were overexpressed, *E. coli* BL21(DE3)-P, in which the *tktA*, *glk*, *aroE*, *aroF*$^{fbr}$ and *aroB* of the aromatic amino acid pathway genes were overexpressed and in *E. coli* BL21(DE3)-P-DK, the genes above were co-expressed.

## Aerobic fermentation of glycerol associated with *glpK* and *glpD* genes enhanced SA accumulation

SA accumulation in *E. coli* BL21 (DE3), *E. coli* BL21 (DE3)-DK, *E. coli* BL21 (DE3)-P, *E. coli* BL21 (DE3)-P-DK and *E. coli* DH5α-T7-P-DK were evaluated in flask cultures with modified M9 medium. SA production in *E. coli* BL21 (DE3)-DK was 72.4 mg/l, which was 2.0-fold higher than that in *E. coli* BL21 (DE3). Moreover, SA accumulation in *E. coli* BL21(DE3)-P-DK reached 200.2 mg/l, which was 2.8- and 2.1-fold higher than that in *E. coli* BL21(DE3)-DK and *E. coli* BL21(DE3)-P, respectively (Fig. 4a, b). While, SA accumulation in *E. coli* DH5α-T7-P-DK was 146.6 mg/l, which was lower than 200.2 mg/l in *E. coli* BL21(DE3)-P-DK. After 48 h of cultivation, glycerol was mostly consumed, with no obvious differences among the samples (Fig. 4c). Therefore, the SA yield of *E. coli* BL21(DE3)-P-DK reached 7.4 mmol SA/mol Gly, which was higher than that of *E. coli* BL21(DE3)-DK and *E. coli* BL21(DE3)-P (Table 2). The culture of *E. coli* BL21 (DE3)-P-DK showed a lower DCW at 3.6 g/l than the 5.3 g/l of *E. coli* BL21 (DE3) (Fig. 4d). Namely, the presence of the plasmids pET-28a-glpD–glpK and pAOC-TGEFB enhanced the yield of SA, but reduced the biomass of *E. coli* BL21 (DE3)-P-DK to some degree.

## Glycerol effectively improved SA production in *E. coli* BL21 (DE3)-P-DK

To investigate the effects of glycerol flux on SA accumulation further, glycerol, glucose, maltose, sucrose, and sorbitol were utilized as different carbon sources. *E. coli*

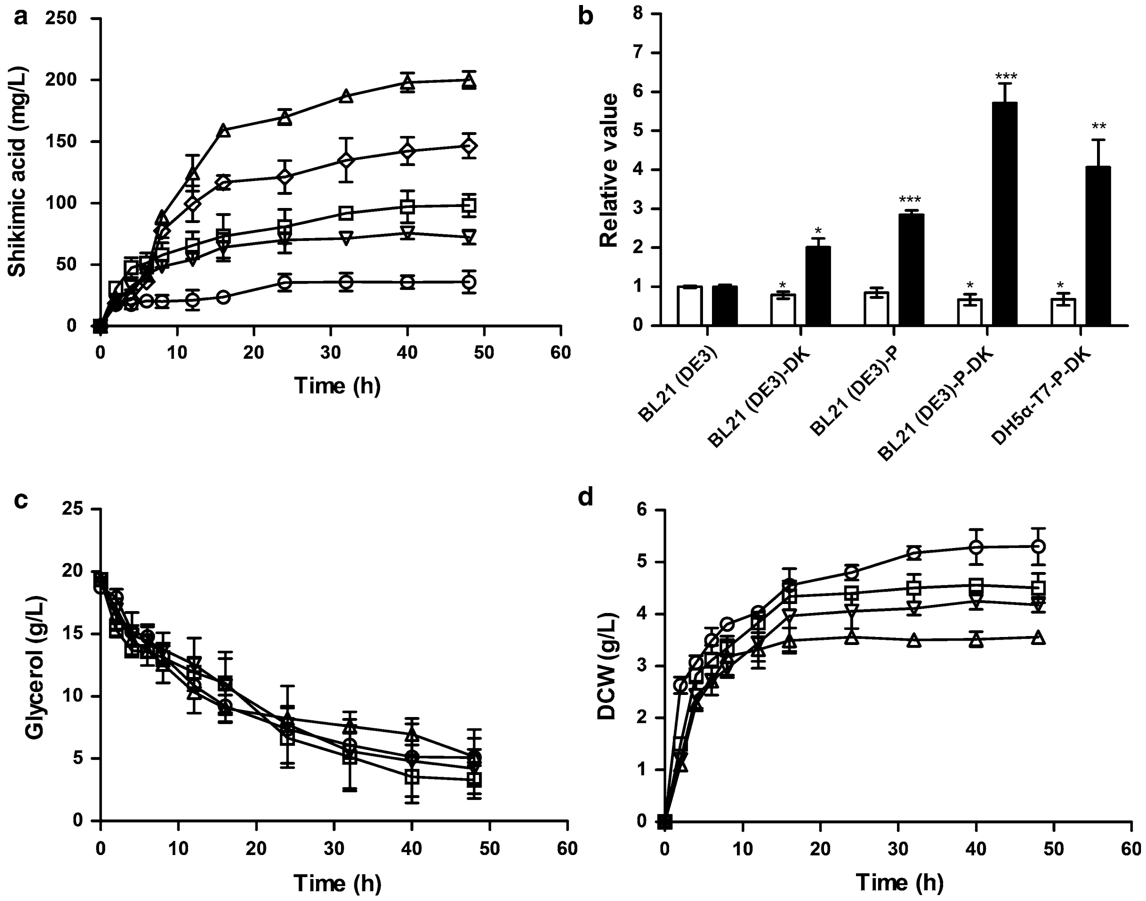

**Fig. 4** Shikimic acid accumulation in *E. coli* BL21(DE3) derivative strains and *E. coli* DH5α-T7-P-DK. **a** Shikimic acid accumulation, **c** glycerol consumption and **d** growth curves. (*open circle*) *E. coli* BL21(DE3); (*open square*) *E. coli* BL21(DE3)-P; (*inverted triangle*) *E. coli* BL21(DE3)-DK; (*triangle*) *E. coli* BL21(DE3)-P-DK; (*diamond symbols*) *E. coli* DH5α-T7-P-DK. **b** Relative SA accumulation in *E. coli* BL21(DE3) derivative strains and *E. coli* DH5α-T7-P-DK: DCW (*open symbols*); SA accumulation (*closed symbols*). *Error bars* represent the standard deviations from triplicate measurements. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with BL21(DE3) group

**Table 2** The SA accumulation, yield and DCW of *E. coli* BL21(DE3) derivative strains

| Strains | Shikimic acid | | DCW | |
|---|---|---|---|---|
| | Accumulation (mg/l) | Yield (mmol SA/mol Gly) | DCW (g/l) | Relative value |
| BL21(DE3) | 35.9 ± 4.9 | 1.4 ± 0.03 | 5.3 ± 0.4 | 1 |
| BL21(DE3)-DK | 72.4 ± 5.6 | 2.5 ± 0.02 | 4.2 ± 0.1 | 0.79 ± 0.06 |
| BL21(DE3)-P | 98.1 ± 9.1 | 3.2 ± 0.06 | 4.5 ± 0.3 | 0.85 ± 0.03 |
| BL21(DE3)-P-DK | 200.2 ± 10.9 | 7.4 ± 0.08 | 3.6 ± 0.1 | 0.67 ± 0.04 |

The values were obtained at the end of batch culture (48 h). The data represent the mean of the triplicate experiments

BL21(DE3) and *E. coli* BL21(DE3)-P-DK grown aerobically in modified M9 complex medium containing carbon source of glycerol (20 g/l) or other carbon sources (20 g/l). The data showed that the highest SA production in *E. coli* BL21(DE3)-P-DK was 200.2 ± 10.9 mg/l in glycerol culture, followed by 142.8 ± 1.2 mg/l in glucose culture, 119.1 ± 5.8 mg/l in maltose culture, 89.9 ± 4.5 mg/l in sucrose culture and 132.9 ± 3.2 in sorbitol (Fig. 5a). In contrast, *E. coli* BL21(DE3) exhibited similar SA production with different carbon sources (Fig. 5b). The biomass of *E. coli* BL21(DE3)-P-DK with different carbon sources was similar (Fig. 5c) but lower than that of *E. coli* BL21(DE3) (Fig. 5d). The SA yield per biomass for *E. coli* BL21(DE3)-P-DK with different carbon sources was then calculated: the highest SA yield was 61.1 mg SA/g glycerol, followed by 42.8 mg SA/g glucose, 34.1 mg SA/g

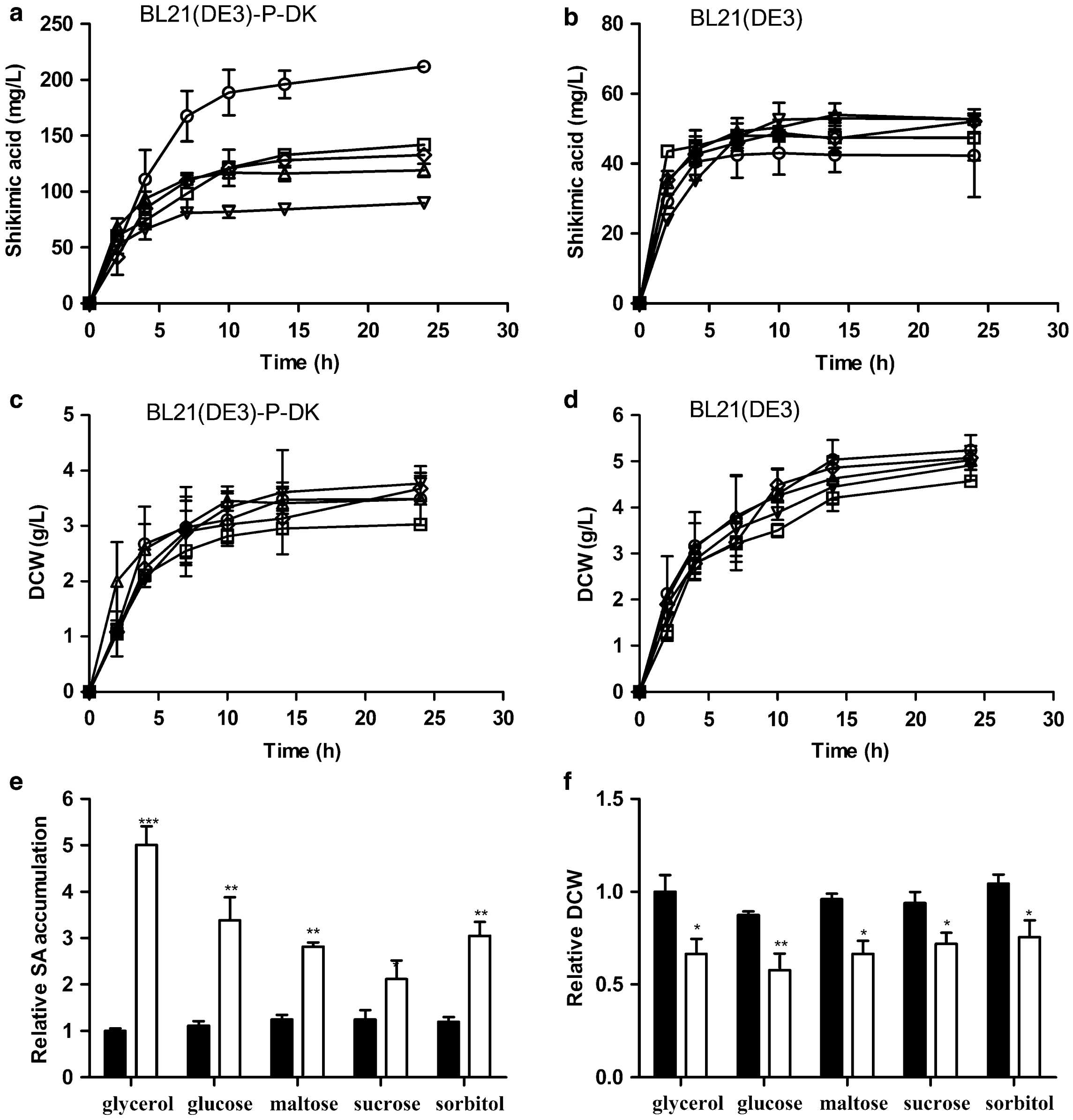

**Fig. 5** Glycerol-enhanced shikimic acid accumulation in strain *E. coli* BL21(DE3)-P-DK. **a**, **b** SA accumulation in *E. coli* BL21(DE3)-P-DK and *E. coli* BL21(DE3). **c**, **d** DCW of *E. coli* BL21(DE3)-P-DK and *E. coli* BL21(DE3). (*open circle*) glycerol; (*open square*) glucose; (*triangle*) maltose; (*inverted triangle*) sucrose; (*diamond symbols*) sorbitol. **e**, **f** The relative values of SA accumulation and DCW using different carbon sources after 24 h of cultivation are shown: *E. coli* BL21(DE3) (*closed symbols*); *E. coli* BL21(DE3)-P-DK) (*open symbols*). *Error bars* represent the standard deviations from triplicate measurements. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with glycerol of BL21(DE3) group

maltose, 23.9 mg SA/g sucrose and 38.1 mg SA/g in sorbitol. It is important to emphasize that *E. coli* BL21 (DE3)-P-DK displayed higher carbon flux toward SA, which was 5-fold higher than that of *E. coli* BL21 (DE3) in glycerol culture. The SA accumulation in *E. coli* BL21(DE3)-P-DK in glucose culture, maltose culture, sucrose and sorbitol culture were 3.0-, 2.2-, 1.7- and 2.8-fold, respectively, higher than that in *E. coli* BL21(DE3) (Fig. 5e, f).

## Discussion

Previous studies have reported successful expression of key enzymes' genes (*aroG*$^{fbr}$, *aroB*, *aroE* and gene) in *E. coli* that can produce 8.9-fold more SA than the parental strain *E. coli* in which *ΔaroK* and *ΔaroL* genes were knocked out (Cui et al. 2014). In the present study, a co-expression plasmid system was constructed with plasmids pAOC-

TGEFB, where the *tktA*, *glk*, *aroE*, *aroF*$^{fbr}$ and *aroB* genes of the aromatic amino acid pathway were overexpressed, and pET-28a-glpD–glpK. In addition, previous studies have mainly focused on the inactivation of the PTS system to channel part of the phosphoenolpyruvate (PEP) not consumed in glucose transport to the aromatic pathway (Rodriguez et al. 2013; Cortés-Tolalpa et al. 2014). Although a PTS$^{-}$ strain accumulated more aromatic compounds in glycerol–glucose culture than in glucose culture, the productivity of aromatic compounds in glycerol culture was higher than that in glucose culture (Martínez et al. 2008; Chen et al. 2012). The strategy of overexpressing *glpK* and *glpD* genes in *E. coli* production strains growing on glycerol has been reported previously (Mazumdar et al. 2010, 2013; Wong et al. 2014), none of these are applied in SA biosynthesis. To investigate the effects of glycerol aerobic fermentation on SA accumulation, increasing glycerol metabolic flux by *glpD* and *glpK* genes overexpressed was carried out to obtain higher SA accumulation. Meanwhile carbon source is one of the main components of culture medium which is a crucial factor for SA production. The effects of various carbon sources on the growth and SA accumulation in *E. coli* strains were explored (Liu et al. 2014). The effect of different carbon source on the SA production was performed in this study. From the data obtained, the *glpD* and *glpK* genes were not successfully expressed in *E. coli* DH5α-T7 (Fig. 2c). The protein III$^{Glc}$ is involved in regulation by inducer exclusion in enteric bacteria and GlpK is inactivated by unphosphorylated III$^{Glc}$ protein. In the *ptsHIcrr* mutant, *E. coli* DH5α-T7, III$^{Glc}$ is present inside the cell and inactivates GlpK (Dean et al. 1990), which could explain why the *glpD* and *glpK* genes were not expressed well in *E. coli* DH5α-T7. Due to the apparent poor expression of *glpD* and *glpK*, we had to change from past host *E. coli* DH5α-T7 to a BL21 (DE3).

In *E. coli* BL21(DE3)-DK, the *glpD* and *glpK* genes were expressed compatibly and SA accumulation reached 72.4 mg/l (Fig. 4a), a 2.0-fold increase over that in *E. coli* BL21(DE3) (Fig. 4b), suggesting that expression of the *glpK* and *glpD* genes could increase glycerol metabolic flux and result in higher SA accumulation. Moreover, SA accumulation in *E. coli* BL21(DE3)-P-DK reached 200.2 mg/l (Fig. 4a), which was 5.6-fold higher than that of *E. coli* BL21(DE3) (Fig. 4b), suggesting increased glycerol metabolic flux toward SA due to the overexpression of *glpD* and *glpK* genes in glycerol dissimilation and *aroE*, *aroB*, *glk*, *tktA*, and *aroF*$^{fbr}$ genes in SA pathway. Previous studies reported that increasing the availability of PEP and erythrose-4-phosphate (E4P) effectively contributes to the accumulation of SA in recombinant *E. coli* (Krämer et al. 2003). Glycerol kinase, encoded by *glpK*, catalyses the conversion of glycerol into glycerol-3-phosphate (G3P). G3P is then converted to dihydroxyacetone phosphate (DHAP) by aerobic glycerol-3-phosphate dehydrogenase, encoded by *glpD*. Thus, the overexpression of the *glpK* and *glpD* genes increases the availability of DHAP, which ultimately enters the Embden–Meyerhof–Parnas pathway and is converted to PEP and E4P (Ahn et al. 2008). Hence, the flux toward SA was increased.

SA accumulation in *E. coli* BL21(DE3)-P-DK reached 200.2 ± 10.9 mg/l in glycerol culture (Fig. 5a), which was the highest in comparison to glucose, maltose and sucrose cultures, suggesting that glycerol is an effective carbon source for SA accumulation. *E. coli* BL21 (DE3)-P-DK displayed higher carbon flux toward SA production, which was 5.0-fold higher than that of *E. coli* BL21 (DE3) in glycerol culture (Fig. 5e). Glucose, maltose and sucrose can be transported or regulated by the PTS system, which consumes PEP (Ahn et al. 2008). In comparison to other carbon sources, however, glycerol enters the glycolytic pathway without consuming PEP. Therefore, part of the PEP not consumed in sugar transport could be converted to DHAP by glycerol kinase and glycerol-3-phosphate dehydrogenase, which are encoded by *glpK* and *glpD*, and then isomerized to glyceraldehyde 3-phosphate (GA3P). GA3P is the substrate of transketolase I (coded by *tktA*), which interconnects the non-oxidative branch of the pentose phosphate pathway and glycolysis (Zou et al. 2004). For these reasons, increasing glycerol metabolic flux can enhance SA accumulation in *E. coli* BL21 (DE3)-P-DK. Previous studies reported that many organisms carry out the fermentative metabolism of glycerol in a 1,3-propanediol-dependent manner under anaerobic conditions. *E. coli* is able to ferment glycerol when appropriate conditions are maintained in the absence of 1,3-propanediol synthesis under aerobic conditions (Yazdani and Gonzalez 2007). These conditions include avoiding the accumulation of the fermentation gas hydrogen, an acidic condition and appropriate medium composition (Dharmadi et al. 2006). Therefore, the accumulation of SA in the cultures simultaneously facilitated the utilization of glycerol.

In the present study, although the overexpression of *glpK* and *glpD* genes led to a notable increase in SA accumulation, it was not high enough to reach the level of previous research (Chen et al. 2012). Further metabolic modifications need to be studied in *E. coli* BL21(DE3)-DK-P, such as blocking SA from the next step in the SA pathway and overexpression of the *galP* gene, which encodes GalP in glycerol transport (Martínez et al. 2008).

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