

Metabolic engineering of *Escherichia coli* to enhance acetol production from glycerol

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Abstract Acetol, a C3 keto alcohol, is an important intermediate used to produce polyols and acrolein. To enhance acetol production from glycerol by *Escherichia coli*, a mutant (HJ02) was constructed by replacing the native *glpK* gene with the allele from *E. coli* Lin 43 and overexpression of *yqhD*, which encodes aldehyde oxidoreductase YqhD that converts methylglyoxal to acetol. Compared to the control strain without the *glpK* replacement, HJ02 had 5.5 times greater acetol production and a 53.4 % higher glycerol consumption rate. Then, glucose was added as a co-substrate to enhance NADPH availability and the *ptsG* gene was deleted in HJ02 (HJ04) to alleviate carbon catabolite repression, which led to a 30 % increase in the NADPH level and NADPH/NADP⁺. Consequently, HJ04 accumulated up to 1.20 g/L of acetol, which is 69.0 % higher than that of HJ02. Furthermore, the *gapA* gene in HJ04 was silenced by antisense RNA (HJ05) to further enhance acetol production. The acetol concentration produced by HJ05 reached 1.82 g/L, which was 2.1 and 1.5 times higher than that of HJ02 and HJ04.

Real-time PCR analysis indicates that glucose catabolism was rerouted from glycolysis to the oxidative pentose phosphate pathway in HJ05.

Keywords Acetol · Glycerol · *GlpK* · NADPH · *GapA*

Introduction

Acetol is an important intermediate used to produce polyols and acrolein, which is to manufacture a reduced dye in the textile industry and a skin tanning agent in the cosmetic industry (Cameron and Cooney 1986; Soucaille et al. 2008a; Mohamad et al. 2011). Acetol can be either synthesized by dehydration of glycerol (Yamaguchi et al. 2010) or produced through dehydrogenation of propylene glycol (Sato et al. 2008). However, the high cost of acetol by chemical processes reduces its industrial applications and markets (Soucaille et al. 2008b). Thus, there is an urgent need for making acetol biologically, which is an environmentally and economically viable approach (Soucaille et al. 2008a). Glycerol, a byproduct of biodiesel (Clomburg and Gonzalez 2013), with high abundance and low price, can be a good carbon source for acetol production.

The acetol biosynthesis pathways of *Escherichia coli* using glycerol as a carbon source with glucose as a co-substrate are shown in Fig. 1. Glycerol is converted into DHAP by a process of phosphorylation (*GlpK*, *glpK*) and dehydrogenation (*GlpD*, *glpD*). DHAP is then converted to methylglyoxal by methylglyoxal synthase (*MgsA*, *mgsA*) (Hopper and Cooper 1972). DHAP is finally transformed to acetol through a NADPH-dependent aldehyde oxidoreductase (*YqhD*, *yqhD*) or aldo-keto reductases (Ko et al. 2005; Soucaille et al. 2008a). The maximum theoretical yield of acetol from glycerol is 1 (1 glycerol + 1 NADPH + NAD⁺ → 1 acetol + 1 NADH + 1 NADP⁺). Hence, glycerol utilization and NADPH availability are two important factors for the acetol production.

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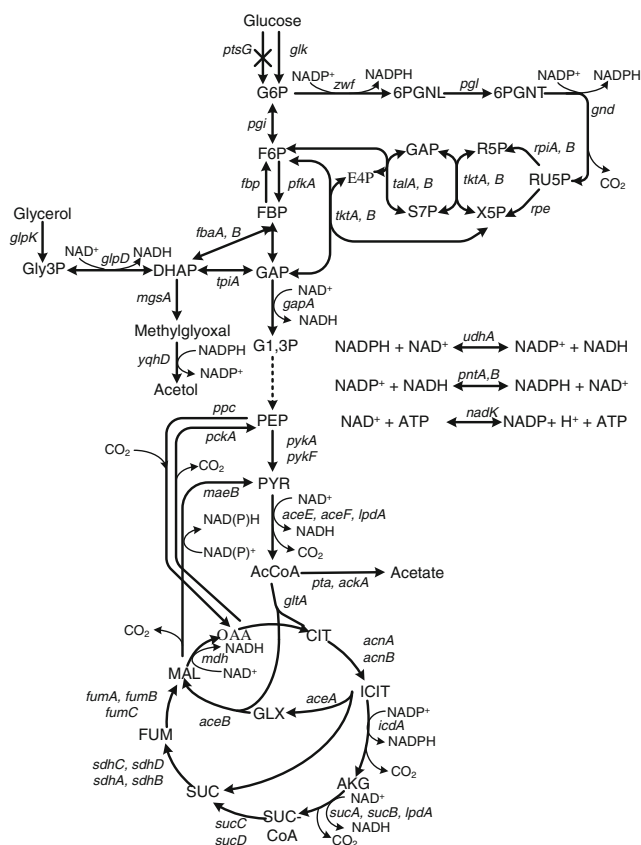


Fig. 1 Metabolic pathways involved in glucose and glycerol with glucose as a co-substrate dissimilations and biosynthesis of acetol in *E. coli*. Broken lines illustrate multiple steps. 6PGNL 6-phospho D-glucono-1,5-lactone, 6PGNL D-gluconate 6-phosphate, AKG 2-Keto-D-gluconate, AcCoA Acetyl-CoA, CIT citrate, DHAP, dihydroxyacetone phosphate, E4P, erythrose 4-phosphate, F6P fructose 6-phosphate, FBP fructose 1,6-bisphosphate, FUM fumarate, G1,3P 1,3-bisphosphoglycerate, G6P glucose-6-phosphate, GAP glyceraldehyde 3-phosphate, GLX glyoxylate, Gly3P glycerol-3-phosphate, ICIT isocitrate, MAL malate, OAA oxaloacetate, PEP phosphoenolpyruvate, PYR pyruvate, R5P ribulose 5-phosphate, Ru5P ribose 5-phosphate, S7P sedoheptulose 7-phosphate, SUC succinate, SUCCoA succinyl-CoA, X5P xylulose 5-phosphate

The main drawback of the glycerol utilization for the production of biochemicals is the relatively low carbon source consumption rate, cell growth, and productivity (Wang and Yang 2013). Glycerol kinase (GlpK) is the rate-limiting enzyme in glycerol metabolism, whose catalytic activity is inhibited allosterically by the fructose 1,6-bisphosphate (FBP) and EIIGlc (Lin 1976). It was reported that glycerol kinase from the strain Lin 43 lost the sensitivity to inhibition by FBP and EIIGlc and consequently improved the cell growth rate and the glycerol consumption rate (Zwaig et al. 1970; Holtman et al. 2001). The sequence of the *glpK22* allele encoding GlpK in strain Lin 43 has a single nucleotide change, Gua-913-Ade, compared to the sequence of the *glpK* gene in *E. coli* BW25113 (Pettigrew et al. 1988). This nucleotide change results in the amino acid change Gly-304 to Ser (G-304-S) (Pettigrew et al. 1988), affecting the catalytic,

regulatory, and conformational properties of GlpK globally (Pettigrew et al. 1996). Neither FBP nor EIIGlc inhibits the mutant enzyme under conditions that produce strong inhibition of the wild-type enzyme, which is sufficient to explain the phenotype of the strain Lin 43 (Pettigrew et al. 1996).

Our previous study demonstrated that overexpression of *yqhD* increased acetol production by 11.4-fold in the Lin 43 strain using glycerol as a sole carbon source (Zhu et al. 2013). As YqhD utilizes NADPH as a co-factor, overexpression of *yqhD* may result in shortage of NADPH (Clomburg and Gonzalez 2011; Jarboe 2011). Several attempts have been devoted to increase NADPH, such as conversion of NADH to NADPH by transhydrogenases, addition of glucose for the regeneration of NADPH, and metabolic engineering to redirect flux towards pentose phosphate (PP) pathway (Lee et al. 2010; Jan et al. 2013; Siedler et al. 2013). The latter two are favored since NADPH is mainly formed in the oxidative branch of the PP pathway. However, there is no flux through the PP pathway under glycerol cultivation, while the flux through PP pathway is $43 \pm 9\%$ of the glucose uptake rate under glucose cultivation (Chubukov et al. 2013). Thus, adding glucose as a co-substrate may be a feasible way to enhance the flux through PP pathway and subsequently increase NADPH availability. Other approaches to improve NADPH production include disruption of *pgi* (phosphoglucose isomerase), *pfkA* (6-phosphofructokinase), and *gapA* (glyceraldehyde 3-phosphosphate dehydrogenase) and overexpression of *zwf* (glucose 6-phosphate-1-dehydrogenase) and *gnd* (6-phosphogluconate dehydrogenase) (Lim et al. 2002; Lee et al. 2010; Siedler et al. 2013). Among these genetic modifications, deletion of *gapA* resulted in a complete cyclization of the PP pathway, thus making the $\Delta gapA$ strain a good candidate of improving NADPH (Siedler et al. 2013).

The aim of this study was to construct a genetically engineered *E. coli* strain to increase glycerol consumption and NADPH for acetol production. Three aspects were investigated to achieve this goal: (1) replacement of *glpK* from the strain Lin 43, (2) addition of glucose as a co-substrate and disruption of glucose PTS, (3) silencing of *gapA*. This work provides an efficient way for acetol production with high production and glycerol utilization.

Materials and methods

Strains and plasmids

The strains and plasmids used in this study are listed in Table 1. The parental strain *E. coli* BW25113 (CGSC 7636) and Lin 43 (CGSC 5511) containing the *glpK22* allele were obtained from the *E. coli* Genetic Stock Center at the Department of Biology, Yale University. The sequence of the *glpK22* gene was found in GenBank under accession no. U41468

Table 1 Strains and plasmids used in this study

Strains and plasmids	Relevant genotype or description	Source or reference
Strains		
BW25113	<i>F⁻ λ⁻ rph⁻¹ ΔaraBAD_{AH33} lacI^f ΔlacZWI16</i>	<i>E. coli</i> Genetic Stock
	<i>rrnB_{T14} ΔrhaBAD_{LD78} hsdR514</i>	Center from Yale University
Lin43	Hfr(PO2A) <i>fhuA22, ΔphoA8, fadL701(T2R), relA1, glpR2(glp^c), pitA10, spoT1, glpK22(fbR), rrnB-2, mcrB1, creC510</i>	<i>E. coli</i> Genetic Stock Center from Yale University
HJ01	BW25113/pCA24N- <i>yqhD</i>	This study
HJ02	HJ01, <i>glpK</i> gene replaced by <i>glpK22</i> from strain Lin 43	This study
HJ03	BW25113/pCA24N, <i>glpK</i> gene replaced by <i>glpK22</i> from strain Lin 43	This study
HJ04	HJ02, <i>ptsG⁻</i>	This study
HJ05	HJ04/pHN1009- <i>gapA</i>	This study
Plasmids		
pCA24N	Cm; <i>lacI^f</i> , pCA24N	Kitagawa et al. (2005)
pCA24N- <i>yqhD</i>	Cm; <i>lacI^f</i> , pCA24N:: <i>yqhD⁺</i>	Kitagawa et al. (2005)
pHN1009	pBR322 <i>ori</i> , <i>Amp^r</i> , <i>lacI^f</i> , <i>P_{trc}</i> , <i>lac_o</i> -PT-MCS	Nakashima and Tamura (2009)
pHN1009- <i>gapA</i>	pHN1009 harboring <i>gapA</i> antisense sequence	This study

(Pettigrew et al. 1996). For deleting and overexpressing genes, the Keio collection (Baba et al. 2006) and the ASKA library (Kitagawa et al. 2005) were used, respectively. The *ptsG* gene in HJ02 was disrupted by P1 phage transduction (Cherepanov and Wackernagel 1995).

Culture medium and growth conditions

All the strains were first precultured in LB medium. The second preculture and the main culture were carried out using M9 minimal medium (per liter 6.81 g Na₂HPO₄, 2.99 g KH₂PO₄, 0.58 g NaCl, and 5.94 g (NH₄)₂SO₄) containing 2 g/L of glucose and 2 g/L of glycerol. The second preculture and the main culture were routinely incubated in 500-mL baffled Erlenmeyer flasks at 37 °C and 220 rpm. The initial pH was around 7. The main culture was carried out in triplicate.

Analytical methods

Bacterial growth was monitored by measuring the optical density of the culture broth at 600 nm (OD₆₀₀) using a spectrophotometer (UV-7504, Xinmao, Shanghai, China). Concentrations of glucose, glycerol, acetate and acetol were measured by high-performance liquid chromatography (model 1260, Agilent, Santa Clara, USA) using a cation-exchange column (HPX-87H, Bio-Rad, Hercules, CA) and a differential refractive index (RI) detector. A mobile phase of 5 mM H₂SO₄ at 0.5 mL/min flow rate was used and the column was operated at 60 °C.

Quantitative real-time reverse transcription PCR (qRT-PCR)

Samples from batch fermentations of strains HJ01, HJ02, HJ04, and HJ05 were collected for RNA extraction at 72 h. Total RNA was isolated using an RNA Extraction Kit (ABigen Corporation, Beijing, China). Contaminating DNA was removed with RNase-free DNase I (ABigen Corporation, Beijing, China). The first-strand cDNA was synthesized using PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara Co. Ltd., Dalian, China). QRT-PCR was performed with the SYBR® Premix Ex Taq™ Kit (Tli RNaseH Plus, Takara Co. Ltd., Dalian, China) on an ABI Stepone Real-Time PCR System (Applied Biosystems, Foster, USA). The primers that were used are listed in Table S1, and the housekeeping gene 16S RNA was used to normalize the gene expression data. The PCR conditions were as follows: 95 °C for 4 min, followed by 35 cycles of denaturation at 95 °C for 15 s, annealing at 57 °C for 15 s, and extension at 72 °C for 20 s. Three samples were analyzed, and each sample was analyzed three times. The data were averaged and presented as the mean ± standard deviation. Significant differences were determined by unpaired two-tailed Student's *t* test, using the SPSS 13.0. Statistical significance was defined as *P* < 0.05.

Quantification of intracellular cofactor levels

Samples from batch fermentations of strains HJ02, HJ03, HJ04, and HJ05 were harvested for NADP⁺/NADPH at 60 h. Intracellular NADP⁺/NADPH were determined by using EnzyChrom NADP⁺/NADPH Assay kit (BioAssay Systems, Hayward, CA), following the manufacturer's instructions.

Data represent the average and standard deviation of measurements from three independent cultures. Statistical analyses were carried out using Microsoft Excel 2013. Multiple comparisons among experimental groups were made by one-way analysis of variance (ANOVA) with the level of significance set at $P < 0.05$.

Results

Replacement of *glpK* enhanced acetol production using glycerol as a carbon source

In order to produce acetol, the strain HJ01 was constructed by the overexpression of *yqhD* in *E. coli* BW25113 to more readily convert methylglyoxal to acetol. Then, the native *glpK* gene in HJ01 was replaced by *glpK22* from the strain Lin 43 to improve glycerol uptake, and the resulting strain was named HJ02.

The glycerol consumption rate and acetol production by HJ01 and HJ02 using glycerol as a sole carbon source at 72 h are shown in Fig. 2. As expected, the HJ02 strain showed a 53.4 % increase in the glycerol consumption rate as compared to HJ01. The base strain HJ01 produced only 0.08 g/L acetol, while the strain HJ02 produced 0.44 g/L acetol, 5.5 times of that in HJ01.

To gain a deeper insight of the effect of replacement of *glpK* on glycerol consumption and acetol production, transcript levels of *glpK* and *mgsA* were measured in HJ01 and HJ02 (Fig. 3a). MgsA converts the glycerol intermediate DHAP to methylglyoxal (Fig. 1). Replacement of the *glpK* gene from the strain Lin 43 resulted in the upregulation of the transcription levels of the *glpK* about 1.7-fold ($P < 0.01$) and consequently increased the glycerol consumption rate. In addition, it was found that *mgsA* was upregulated by 2.5-fold in HJ02 ($P < 0.01$).

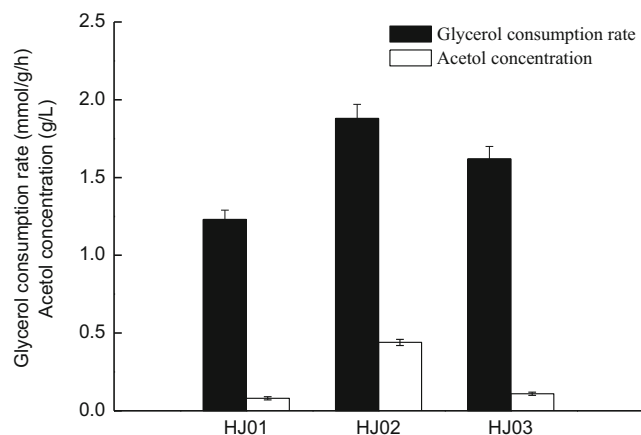


Fig. 2 The glycerol consumption rate and acetol production by HJ01 and HJ02 using glycerol as a sole carbon source at 72 h

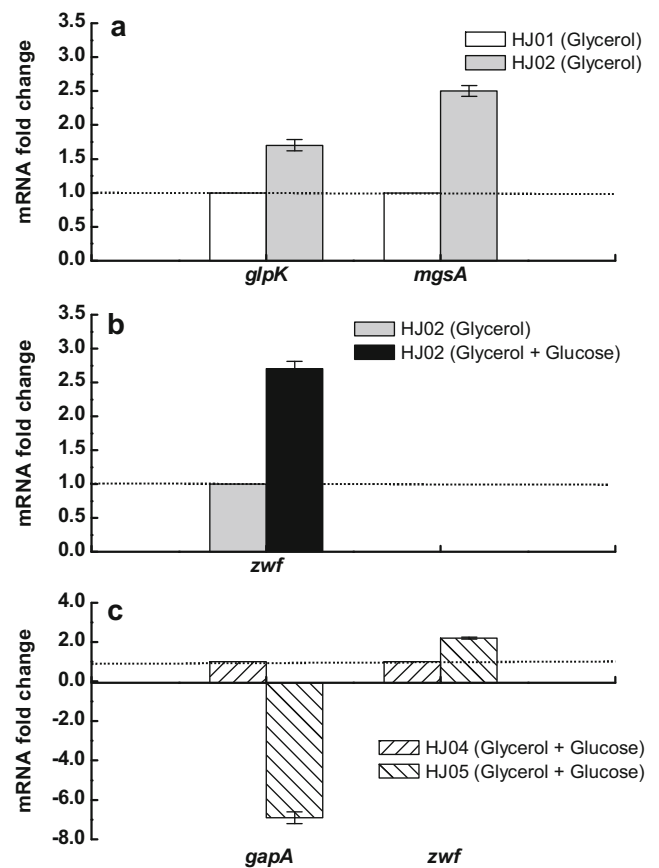


Fig. 3 Fold changes of transcription levels of selected genes in **a** HJ02 using glycerol as a sole carbon source compared to HJ01 using glycerol as a sole carbon source, **b** HJ02 using glycerol and glucose as carbon sources compared to HJ02 using glycerol as a sole carbon source, and **c** HJ05 using glycerol and glucose as carbon sources compared to HJ04 using glycerol and glucose as carbon sources

The aforementioned manipulations did not permit the product, acetol, to accumulate to a high final concentration. The reason might be that the co-factor NADPH available could not meet the needs of YqhD in HJ02, which subsequently resulted in small amount of acetol production. To confirm this hypothesis, the NADPH levels and NADPH/NADP⁺ ratios were measured in HJ02 and the control strain HJ03 (HJ02 harboring an empty plasmid of pCA24N). As expected, the overexpression of *yqhD* lowered NADPH level by 35.8 % ($P < 0.01$) and NADPH/NADP⁺ level by 25.1 % ($P < 0.05$) in HJ02, respectively (Table 2). The result suggests the need for further study of improving NADPH levels in the acetol production strains.

Disruption of *ptsG* enhanced acetol production using glycerol and glucose as carbon sources

Since glucose can serve as substrate for the regeneration of NADPH (Siedler et al. 2013), 2 g/L of glucose was added into the media. Figure 4a shows the batch fermentation characteristics of *E. coli* HJ02 using glucose and glycerol as carbon

Table 2 Comparison of NADPH, NADP⁺ and NADPH/NADP⁺ of the engineered strains

Strain	Carbon source	NADPH (nmol/mg)	NADP ⁺ (nmol/mg)	NADPH/NADP ⁺
HJ02	Glycerol	0.31 ± 0.01	0.49 ± 0.02	0.63 ± 0.03
HJ02	Glycerol + glucose	0.54 ± 0.02	0.59 ± 0.02	0.92 ± 0.05
HJ03	Glycerol	0.43 ± 0.01	0.53 ± 0.02	0.81 ± 0.04
HJ04	Glycerol + glucose	0.70 ± 0.02	0.58 ± 0.02	1.21 ± 0.04
HJ05	Glycerol + glucose	0.93 ± 0.03	0.66 ± 0.02	1.41 ± 0.06

sources. Glucose was the first to be consumed, while glycerol was begun to consume upon glucose depletion (24 h). The sequential utilization of glucose and glycerol is due to carbon catabolite repression (CCR) (Deutscher et al. 2006). The acetol concentration was increased to 0.71 g/L, 61 % higher than using glycerol as a sole carbon source. Since HJ02 did not produce acetol using glucose as a sole carbon source, the acetol was all from glycerol (data not shown). In addition, upregulation of *zwf* ($P < 0.01$) encoding the glucose-6-phosphate dehydrogenase (G6PDH) in the first step of oxidative PP pathway implied that the presence of glucose plays an important role in inducing the PP pathway (Fig. 3b). Furthermore, NADPH level and NADPH/NADP⁺ ratio were enhanced by 1.72- and 1.43-fold by adding glucose, respectively (Table 2).

Chin et al. (2009) found that more than 60 % of the available energy in the form of NADPH resulting from glucose oxidation was dissipated. In our case, glucose was totally depleted before glycerol was consumed; therefore, the NADPH generated by glucose may be utilized by respiration other than YqhD. Thus, simultaneous consumption of glucose and glycerol should improve the efficacy of NADPH utilization by YqhD and further facilitate the increase of acetol production. To test this, *ptsG*, the center player of CCR was deleted in HJ02, yielding the strain HJ04. The gene *ptsG* encodes the membrane-bound protein (EIICB^{Glc}), which is specific for glucose transport (Gosset 2005). As shown in Fig. 4b, with inactivation of *ptsG*, the simultaneous consumption of glucose and glycerol can be attained. The acetol concentration was increased to 1.20 g/L, 69.0 % higher than that of HJ02. Moreover, the yield of acetol from glycerol was also enhanced, more than 2.5-fold higher than that of HJ02. Interestingly, we found that the NADPH level and NADPH/NADP⁺ ratio were both increased around 30 % in the *ptsG* mutant compared to the control using glucose-glycerol mixture ($P < 0.05$ and $P < 0.01$, respectively) (Table 2).

Silencing of *gapA* enhanced acetol production using glycerol and glucose as carbon sources

Deletion of *gapA* in *Corynebacterium glutamicum* resulted in a complete cyclization of the PP pathway, which further improved NADPH supply (Siedler et al. 2013). However, the deletion of *gapA* in *E. coli* severely impaired the cell growth

(Seta et al. 1997). The RNA silencing method described by Nakashima and Tamura (2009) is an alternative way to down-regulate the transcription level of *gapA*, allowing for growth in *E. coli* while enhancing PP pathway. Therefore, HJ05 was constructed by silencing of *gapA* from HJ04, and batch fermentation characteristics of this strain using glycerol-glucose mixture were studied (Fig. 4c).

As compared to HJ04, silencing of *gapA* reduced cell growth, glycerol consumption, and glucose consumption in HJ05 (Fig. 4c). Critically, the acetol concentration reached 1.82 g/L in HJ05, 2.1 times higher than that of HJ02, 1.5 times higher than that of HJ04. The acetol yield on glycerol was also increased to 0.25 g/g in HJ05, 2.8-fold higher than that of HJ02, 1.8-fold higher than that of HJ04.

In order to understand the effect of silencing of *gapA*, transcript levels of *gapA* and *zwf* were compared in HJ04 and HJ05 (Fig. 3c). The downregulation of the *gapA* was about

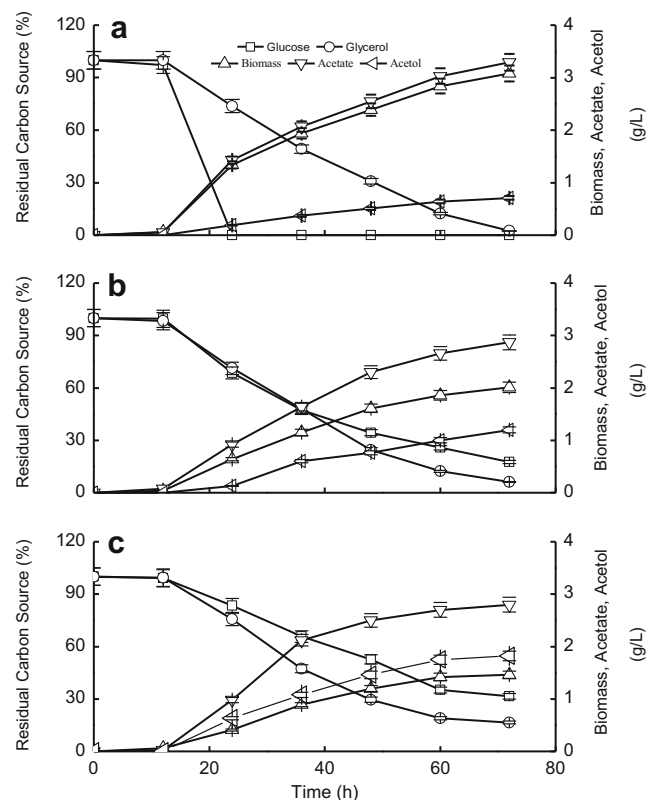


Fig. 4 Batch fermentation characteristics of *E. coli* HJ02 (a), HJ 04 (b), and HJ05 (c) using a mixture of glucose and glycerol as carbon sources

6.9-fold ($P < 0.01$) in HJ05. The transcription level of *zwf* was upregulated by 2.2-fold ($P < 0.01$). The NADPH level in HJ05 was 72.4 % higher than that of HJ02 and 32.3 % higher than that of HJ04 ($P < 0.01$ and $P < 0.05$, respectively) (Table 2). The NADPH/NADP⁺ ratio in HJ05 also showed an increase as compared to HJ02 and HJ04 ($P < 0.01$ and $P < 0.05$, respectively).

Discussion

Glycerol has become a readily available and inexpensive carbon source since it is a byproduct of biodiesel production (Almeida et al. 2012; Clomburg and Gonzalez 2013). Therefore, it is attractive to consider using fermentative production of biochemicals based on glycerol with metabolically engineered *E. coli* cells (Clomburg and Gonzalez 2013; Mazumdar et al. 2013; Gottlieb et al. 2014). However, the consumption rate of glycerol by *E. coli* is lower compared to glucose, leading to the reduced cell growth and productivity (Wang and Yang 2013). Since the GlpK-GlpD pathway mediates glycerol dissimilation under aerobic conditions, the strategy of overexpressing the *glpK* and *glpD* genes in *E. coli* strains to increase glycerol flux has been reported previously (Mazumdar et al. 2013; Wong et al. 2014; Yang et al. 2014). In this study, the effect of the replacement of *glpK* from the strain Lin 43 on acetol production was investigated. In HJ02, the glycerol consumption rate was higher than HJ01, and the acetol accumulation was 5.5 times of that in HJ01, showing that replacement of *glpK* from the strain Lin 43 increases the glycerol metabolic flux and result in higher acetol production (Fig. 2). Furthermore, the upregulation of *glpK* in HJ02 implies that the inhibition of GlpK by FBP and EIIA^{Glc} to GlpK might be relieved in HJ02. The strain Lin 43, which is insensitive to inhibition by FBP and EIIA^{Glc}, synthesized excessive methylglyoxal during unregulated glycerol metabolism (Freedberg et al. 1971). The determining factor in the production of methylglyoxal appears to be a high level of the kinase, GlpK (Freedberg et al. 1971). We hypothesize that the strain HJ02 possessing the same *glpK* as the strain Lin 43 may also accumulate methylglyoxal. Furthermore, induction of *mgsA* in HJ02 appears to translate into more methylglyoxal for conversion to acetol, which was consistent with excessive accumulation of methylglyoxal in the strain Lin 43. Usually, overexpression of *mgsA* was required to improve the flux channeled to methylglyoxal pathway to improve methylglyoxal availability (Clomburg and Gonzalez 2011; Jain et al. 2014). Our result indicates that HJ02 increased the flux to methylglyoxal as well as acetol without the need of extra gene copies of *mgsA*. In addition, methylglyoxal is a very toxic metabolite and arrests growth of *E. coli* at sub-millimolar concentrations (Booth et al. 2003). The strain Lin 43 was killed when exposed to glycerol during growth on succinate or casein amino

acids (Zwaig et al. 1970). In our system, overexpression of *mgsA* in HJ02 showed a strong growth defect (data not shown). This may be because artificially increasing *mgsA* caused excess methylglyoxal to be produced faster than it could be transformed to acetol. Thus, the production of methylglyoxal should be tightly controlled. Hence, this approach provides an alternative method to enhance *mgsA* expression at moderate levels by replacement of *glpK*, reducing the metabolic burden resulted from *mgsA* overexpression.

Besides direct manipulations of the enzymes that are involved in the pathway for the

target metabolites, cofactor engineering is a powerful tool in the field of metabolic engineering to increase productivity (Lee et al. 2010; Wu et al. 2014). In this work, we demonstrated that NADPH availability and acetol production had a strong correlation with each other. Major resources of NADPH in *E. coli* include isocitrate dehydrogenase in the TCA cycle (Reeves et al. 1968), the transhydrogenase system (Cui et al. 2014), and PP pathway (Sauer et al. 2004). Previous studies reported that the PP pathway is the main contributor of NADPH (Sauer et al. 2004). However, when cells were grown on glycerol, the flux through the PP pathway may be insufficient (Chubukov et al. 2013; Marr 1991). Thus, we added glucose as a co-substrate to enhance the flux through the PP pathway to increase NADPH supply. RT-PCR data showed that the presence of glucose upregulated *zwf* and greatly influenced NADPH and NADPH/NADP⁺, which in turn enhanced acetol biosynthesis. The result is in agreement with Lee's report (2010) that the expression level of *zwf* was significantly upregulated and resulted in increased NADPH and NADPH/NADP⁺ when disrupting *pgi*. The data indicate that glucose has the ability to elevate the NADPH supply for the acetol production. Furthermore, deletion of *ptsG* resulted in increased NADPH level and NADPH/NADP⁺ ratio and subsequently increased the acetol production. Martínze et al. (2008) reported that the strains lacking of PTS are capable of cointilizing glucose and other carbon sources. In our results, simultaneous consumption of glucose and glycerol was attained by the $\Delta ptsG$ strain, HJ04, indicating carbon catabolite repression is relieved. The absence of carbon catabolite repression helps HJ04 to lose glucose control of glycerol utilization with prolonged glucose consumption time, avoiding NADPH dissipation by respiration. This result indicates that simultaneous consumption of glycerol and glucose by disruption of the *ptsG* genes is beneficial to improve the efficacy of NADPH utilization by YqhD, contributing to higher acetol production.

Theoretically, disruption of *gapA* enables a yield of 12 mol NADPH per mole of glucose 6-phosphate by complete recycling of fructose 6-phosphate and glyceraldehydes 3-phosphate through the oxidative PP pathway (Kruger and von Schaewen 2003). Siedler et al. (2013) obtained a $\Delta gapA$ mutant of *C. glutamicum* with a high yield of chiral (R)-

methyl 3-hydroxybutyrate from glucose (7.9 mol/mol). As the disruption of *gapA* in *E. coli* hindered the cell growth (Seta et al., 1997), the RNA silencing method was more feasible to reduce GapA activity. The *gapA* silencing strain HJ05 reached the 1.82 g/L acetol, which was the highest in comparison to HJ02 and HJ04. Compared to HJ04, the downregulation of *gapA* together with improved acetol production suggests that carbon flux was diverted from lower glycolysis to methylglyoxal pathway. The upregulation of *zwf* and elevated NADPH level indicated that glucose catabolism was rerouted from glycolysis to the oxidative PP pathway. The above results indicate silencing of *gapA* is advantageous for improving NADPH by redistributing the glycolysis flux.

In this study, we showed that replacement of *glpK* from the strain Lin 43 resulted in an increased glycerol consumption rate, as well as an increase in the supply of methylglyoxal, the substrate of YqhD. Next, adding glucose as a co-substrate elevated the NADPH supply for acetol production. Furthermore, disruption of glucose PTS gene *ptsG* improved the efficacy of NADPH utilization by YqhD. Finally, silencing of *gapA* led to a complete cyclization the PP pathway, obtaining the highest NADPH among these strains.

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Conflict of interest The authors declare that they have no competing interests.

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