Bioresource Technology 101 (2010) 4157–4161

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Engineering of glycerol utilization pathway for ethanol production by *Saccharomyces cerevisiae*

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ARTICLE INFO

Article history:
Received 30 October 2009
Received in revised form 11 January 2010
Accepted 14 January 2010
Available online 9 February 2010

Keywords:
Glycerol
Fermentation
Saccharomyces cerevisiae
Ethanol

ABSTRACT

Saccharomyces cerevisiae was metabolically engineered to improve ethanol production from glycerol. High rates of glycerol utilization were achieved by simultaneous overexpression of glycerol dehydrogenase (*Gcy*) and dihydroxyacetone kinase (*Dak*), which are the enzymes responsible for the conversion of glycerol to glycolytic intermediate dihydroxyacetone phosphate. As a result, ethanol production in YPH499 (p*GcyaDak*) was about 2.4-fold higher than wild strain. We have also successfully expressed a glycerol uptake protein (*Gup1*). The overall ethanol production in strain YPH499 (p*GcyaDak*, p*GupCas*) was 3.4-fold more than in wild strain, with about 2.4 g L^{-1} ethanol produced. These experimental results confirmed our metabolic pathway strategies which improve the production of ethanol.

1. Introduction

Biodiesel is produced by a transesterification reaction using vegetable oils or animal fats and an alcohol, a process that inevitably generates large amounts of glycerol as a by-product. The availability of crude glycerol is predicted to increase in the next years because of the tremendous growth in the production of biodiesel worldwide. The conversion of value-added products especially using cheaper resources such as glycerol could develop the biofuel industry becoming more economically viable.

Although may microorganisms can metabolize glycerol in the presence of external electron acceptors (respiratory metabolism), few are able to do so fermentatively (Yazdani and Gonzalez, 2007). Recently, engineered *Escherichia coli* strains have been reported for the efficient conversion of crude glycerol into ethanol (Shams Yazdani and Gonzalez, 2008).

Saccharomyces cerevisiae is able to utilize glycerol as a sole source of carbon. Glycerol fermentation in these organisms is mediated by a two-branch pathway, which results in the synthesis of glycolytic intermediate dihydroxyacetone phosphate (DHAP) and fermentation product ethanol. Respiratory (*GUT1–GUT2*) and fermentative (*Gcy–Dak*) routes mediated the conversion of glycerol to glycolytic intermediates. The respiratory pathway involves a glycerol kinase encoded by *GUT1* (Pavlik et al., 1993; Krantz et al., 2009), and a mitochondrial glycerol 3-phosphate dehydrogenase encoded by *GUT2*. In the fermentative pathway glycerol is first converted into dihydroxyacetone by a glycerol dehydrogenase (*Gcy*) and further to dihydroxyacetone phosphate by a dihydroxyacetone kinase (*Dak*) but this pathway is not well described in *S. cerevisiae*.

Physiological characterization of glycerol uptake in *S. cerevisiae* has shown that glycerol enters the cell by at least two different mechanisms depending on the growth conditions. When glucose is present, glycerol can enter the cells by facilitated transport, probably through the glycerol channel, encoded by the *Fps1* (Sutherland et al., 1997; Beese et al., 2009). However, in ethanol-, glycerol- and acetate-grown cells, a proton symport system ensures the uptake of glycerol (Kayingo et al., 2009; Abe et al., 2008). The *Gup1* (glycerol uptake protein) is essential for proper growth on glycerol, as well as for glycerol-mediated recovery from salt stress. It was also necessary for the active uptake of glycerol taking place via the proton symport system (Abe et al., 2008; Lages and Lucas, 1997).

In this study, we produced ethanol in metabolically engineered *S. cerevisiae* to improve the carbon flux to the target metabolite. Overexpression of three genes was conducted to improve ethanol productivity. The first was the overexpression of *Gcy* and *Dak*, which play roles in the synthesis of DHAP. Next was the overexpression of *Gup1*, which plays a role in the uptake of glycerol. Through these gene manipulations, a total of 2.4 g L^{-1} of ethanol was produced by *S. cerevisiae*. Here, we describe the engineering of *S. cerevisiae* for fermentative metabolism of glycerol and its application for ethanol production.

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doi:10.1016/j.biortech.2010.01.066

2. Methods

2.1. Strains and plasmids

E. coli DH5α was used for sub-cloning. The YPH499 499 (*MATa ura3-52 lys2-801_amber ade2-101_ochre trp1-Δ63 his3-Δ200 leu2-Δ1*) (Clontech Laboratories Inc.) strain was genetically modified to produce ethanol (Table 1). The construction of plasmids used in this study is illustrated in Fig. 1a. The expression vector p*GcyaDak* was constructed as follows. The coding region of the *Gcy, Dak* gene was subjected to PCR amplification using genomic DNA of *S. cerevisiae* YPH499 as template and *Gcy, Dak* primers (Table 1). The restriction enzyme sites BamHI, SalI and SpeI, ClaI were introduced through the forward and reverse primers, respectively, to facilitate cloning of the PCR product in the expression vector p*GcyaDak*. Standard recombinant DNA procedures were used for gene cloning, plasmid isolation, and electroporation. Manufacturers' protocols and standard methods were followed for DNA purification (Qiagen, Valencia, CA), restriction endonuclease digestion (New England Biolabs, Ipswich, MA), and DNA amplification (Invitrogen, Carlsbad, CA). To construct the expression vector for the *Gcy, Dak* genes, a pEST-TRP vector was purchased from Stratagene (San Diego, USA).

2.2. Sequential gene integrations

The coding region of the *Gup1* gene was amplified by PCR using genomic DNA of *S. cerevisiae* YPH499 as template and *Gup1* primers (Table 1). Plasmid p*GupCas* contains the *S. cerevisiae Gup1* gene between the *S. cerevisiae Gal1* promoter and *CYC1* terminator. YIP-5 is an integrating vector carrying the reusable *URA3* blaster cassette for selection and an *S. cerevisiae* target site for integration. Expression vectors carrying *Gup1* were constructed using YIP-5 as the backbone. The *Gup1* cassette (*Gup1* preceded by the *Gal1* promoter and followed by the *CYC1* terminator) was amplified using plasmid DNA of p*GUP1* and *Gup cas* primer (Fig. 1b). The *Gal1p–Gup1–CYC1t* cassette was inserted into the BamHI site on YIP-5. The resulting integrating vectors, p*GupCas* (8.54 kb), contained the sequence for homologous recombination, the *URA3* blaster cassette (Kaneko et al., 2009) for selection, and *Gup1* under the control of the *Gal1* promoter. The plasmid of p*GupCas* was linearized by SalI digestion and transformed into strains. Transformation of the integration plasmid p*GupCas* into *S. cerevisiae* was carried out by the lithium acetate method by using a YEASTMAKER yeast transformation system (Clontech Laboratories Inc.). Yeast transformants were selected on SD agar plate.

Table 1
Strain, Plasmids, and primer used in this study.

Strain/plasmid/ primer	Description/genotype/sequence
Strains	
YPH499	*MATa ura3-52 lys2-801_amber ade2-101_ochre trp1-Δ63 his3-Δ200 leu2-Δ1*
Plasmids	
p*Gcy*	*S. cerevisiae Gcy gene under control of Gal1*
p*Dak*	*S. cerevisiae Dak gene under control of Gal10*
p*GcyaDak*	*S. cerevisiae Gcy and Dak gene under control of Gal*
p*Gup*	*S. cerevisiae Gup1 gene under control of Gal10*
p*GupCas*	*S. cerevisiae Gup1 cassette gene under control of Gal10*
Primer	
Gcy	ggatccatgcctgctactttacatgattct gtcgacatacttgaatacttcgaaaggag
Dak	actagtatgtccgctaaatcgtttgaagtc atcgatatacaaggcgctttgaaccccctt
Gup	gaattcatgt cgctgatcag catcctg actagtccag cattttaggt aaattccgtg
GupCas	ggatccatgt cagcatttta ggtaaattcc gtg ggatccataa tgtcgctgat cagcatcctg tct

2.3. Media and cultivation conditions

Luria–Bertani medium (0.5% yeast extract, 1% tryptone, 1% NaCl) was used to culture *E. coli*, and 50 μg ml^{-1} of ampicillin was added for selecting transformants. Yeast was aerobically cultivated at 30 °C in synthetic medium (0.17% of YNB (yeast nitrogen base without amino acid) per liter with 0.13% TRP dropout amino acid to which 2% glucose per liter was added as the sole carbon source (SD medium) for selecting transformants. YPD medium (1% yeast extract, 2% peptone, 2% glucose) was used for culture. SG medium (2% galactose, 0.17% yeast YNB and 0.13% TRP drop out amino acid) and YPG (1% yeast extract, 2% peptone, 2% galactose) were used for inducing gene expression. SG-glycerol (0.02% galactose, 0.17% yeast YNB and 0.13% TRP drop out amino acid, 2% glycerol) was used for fermentation. The fermentation experiments were performed in triplicate.

2.4. Analytical methods

Cell growth was monitored by measuring the optical density at 550 nm. Fermentation samples were thawed and spun down to separate cells from the supernatant. The supernatant was stored at −20 °C for gas chromatography. The gas chromatograph (model GC7890; Agilent, Santa Clara, CA) fitted with a flame ionization detector and a DB-WAXetr column was operated under the following conditions: temperatures of column and injector, 120 and 250 °C, respectively; Helium gas flow rate, 0.6 ml min^{-1}. The supernatant was then tested for glycerol content. The concentration of glycerol was analyzed and quantified using the glycerol reagent from Sigma (Cat. # F6428). All samples were analyzed at 20 °C and stored at −20 °C.

2.5. Enzyme activities

The activity of glycerol dehydrogenase and dihydroxyacetone kinase was measured as previously described (Gonzalez et al., 2008). Enzyme activities are reported as μmoles of substrate/min/mg of cell protein.

3. Results

3.1. Metabolic engineering strategy to efficiently convert glycerol into DHAP

It was previously reported that a two-enzyme pathway was responsible for the conversion of glycerol to glycolytic intermediates: (i) the *GUT1–GUT2* route under respiratory conditions and (ii) the *Gcy–Dak* route under fermentative conditions in *S. cerevisiae*. Our key metabolic engineering strategy was to increase the rate of glycerol utilization which involves the overexpression of the enzymes *Gcy* and *Dak*.

The plasmid map of the p*GcyaDak* vector that contains the *Gcy* gene under a *GAL1* promoter and the *Dak* gene under a *GAL10* promoter is shown in Fig. 1a. Ethanol production was expected to be increased via the expression of *Gcy* and *Dak* genes owing to the increase of the carbon flux to DHAP. When the YPH499 (pESC-TRP) and YPH499 (p*GcyaDak*) strains were grown for 48 h in SD media, glycerol consumption in YPH499 (p*GcyaDak*) was much higher than with YPH499 (pESC-TRP) (Fig. 2). Crude extracts of the negative control strain YPH499 (pESC-TRP) showed low enzyme activities, while crude extracts of YPH499 (p*GcyaDak*) showed high

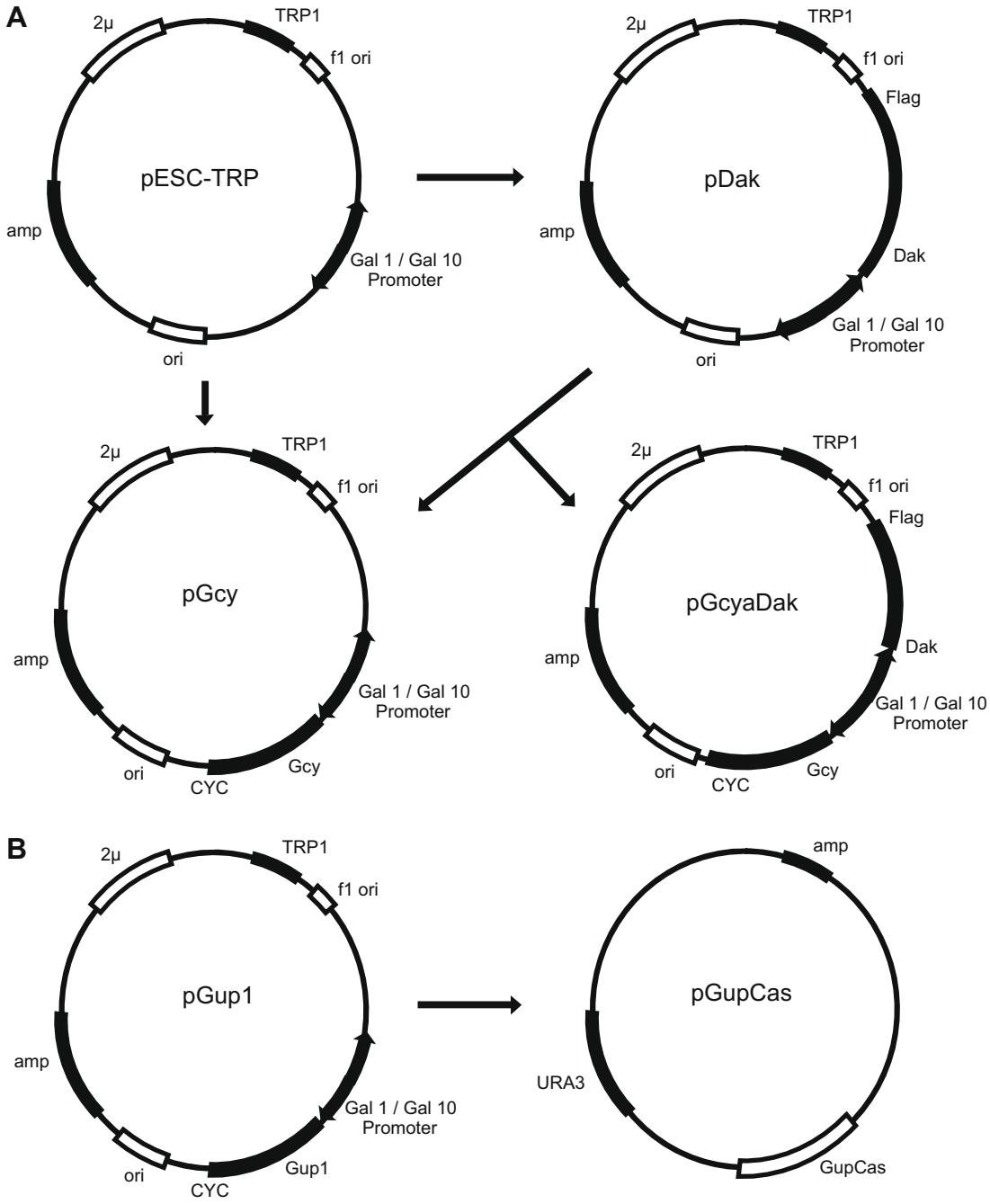

Fig. 1. Diagram illustrates the construction of plasmids used in this study. Plasmids p*GcyaDak* (A) and p*GupCas* (B) are described in the respective figure.

specific activities of glycerol dehydrogenase (1.08 U mg^{-1}). Dihydroxyacetone kinase activity was detected in crude extracts of YPH499 (p*GcyaDak*) (0.35 U mg^{-1}), but was not detected in YPH499 (pESC-TRP) (<0.01 U mg^{-1}) (Table 2).

The studies described above also provided an initial assessment on how cell growth and glycerol fermentation are affected by the overexpression of p*GcyaDak*. The overall glycerol consumption in YPH499 (p*GcyaDak*) strain was 2.4-fold more than that in YPH499 (pESC-TRP).

3.2. Overexpression of Gup1 for the uptake of glycerol

In ethanol-, glycerol- and acetate-grown cells, a *Gup1 gene* was necessary for the active uptake of glycerol taking place via the proton symport system. In order to increase glycerol uptake, a homologous recombination method was used to transform integration plasmid p*GupCas* into *S. cerevisiae*. The integration of p*GupCas* was confirmed by PCR amplification with *GupCas* primers. The ethanol production was also improved when p*GcyaDak* with p*GupCas* was overexpressed (Fig. 3). Overexpression of p*Gup1* increased ethanol production by a small but insignificant fraction (data are not shown).

YPH499 (p*GcyaDak*, p*GupCas*) produced 2.4 g L^{-1} ethanol, whereas YPH499 (pESC-TRP) produced 0.69 g L^{-1} after 96 h of cultivation. The glycerol consumption rate increased compared to YPH499 (p*GcyaDak*) after 12 h of cultivation. The concentration of ethanol increased about 3.4-fold compared to YPH499 (pESC-TRP) (Fig. 3). We have also overexpressed the p*GupCas* with p*GcyaDak*, which led to slightly increased glycerol consumption (Fig. 4).

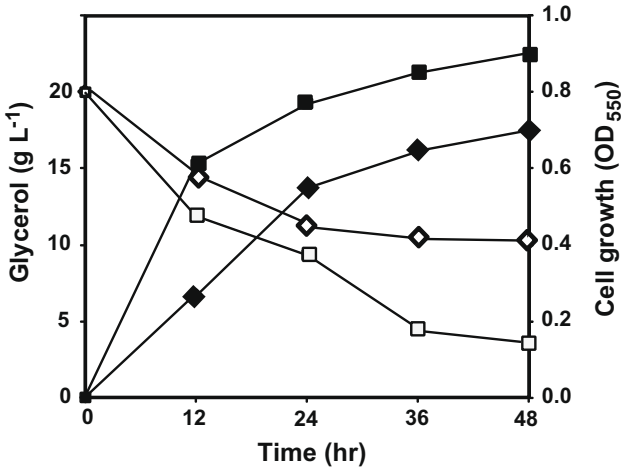

Fig. 2. Effect of expression level on cell growth (filled symbol) and glycerol fermentation (empty symbol). Symbols: diamond, YPH499 (pESC-TRP); square, YPH499 (p*GcyaDak*).

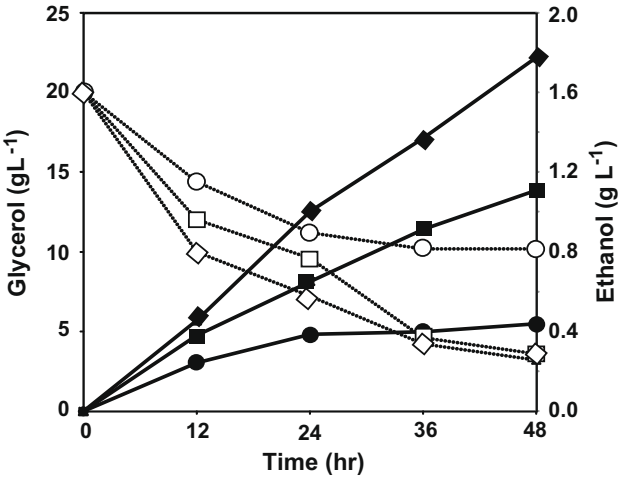

Fig. 4. Conversion of crude glycerol to ethanol by YPH499 strains. Data are shown for the concentration of glycerol (empty symbol) and ethanol (filled symbol) in fermentations conducted using crude glycerol. Symbols: circles, YPH499 (pESC-TRP); squares, YPH499 (p*GcyaDak*); diamonds, YPH499 (p*GcyaDak*, p*GupCas*).

Table 2
Specific activities of glycerol dehydrogenase and dihydroxyacetone kinase in recombinant *S. cerevisiae* strains.

S. cerevisiae strain	Specific activities (μmol min^{-1} mg^{-1}) of:	
	Glycerol dehydrogenase	Dihydroxyacetone kinase
YPH499 (pESC-TRP)	0.01	<0.01
YPH499 (p*GcyaDak*)	1.08	0.35
YPH499 (*pGcyaDak*, *pGupCas*)	0.70	0.12

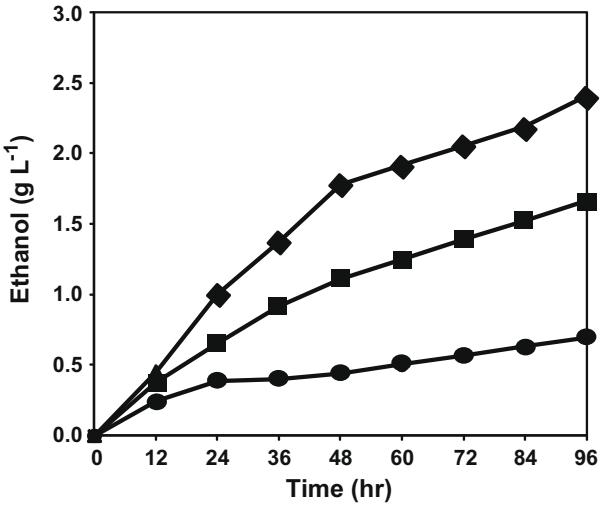

Fig. 3. Production of ethanol by strains of YPH499 (p*GcyaDak*, *pGupCas*). Symbols: circles, YPH499 (pESC-TRP); squares, YPH499 (p*GcyaDak*); diamonds, YPH499 (p*GcyaDak*, p*GupCas*).

3.3. Ethanol production by recombinant S. cerevisiae on glycerol

Given our success on improving the rate of glycerol utilization through p*GcyaDak*, p*GupCas* overexpression, we evaluated the performance of an integrated biocatalyst for the production of ethanol. High rates of glycerol utilization and product synthesis were achieved by simultaneous overexpression of glycerol dehydrogenase (*Gcy*), dihydroxyacetone kinase (*Dak*) and high rates of glycerol uptake by simultaneous overexpression of p*GupCas*.

Fig. 4 shows complete profiles for strains YPH499 (pESC-TRP), YPH499 (p*GcyaDak*) and YPH499 (p*GcyaDak*, p*GupCas*). While a culture of strain YPH499 (p*GcyaDak*) consumed almost 16.4 g L^{-1} of glycerol at 48 h, the reference strain YPH499 (pESC-TRP) only consumed 10 g L^{-1} glycerol over a period of 48 h (Fig. 4). This translates into a 1.6-fold increase in the rate of glycerol consumption. Faster consumption of glycerol was the result of both faster growth and higher specific rate of glycerol consumption. The YPH499 (p*GcyaDak*) with p*GupCas*, also shows enhanced ethanol production. When grown under YPH499 (pESC-TRP) conditions and using high concentrations of glycerol, YPH499 (p*GcyaDak*, p*GupCas*) has efficiently converted glycerol to ethanol, in comparison to the wild type. Fig. 4 shows the ethanol production in medium containing 20 g L^{-1} glycerol using strain YPH499 (p*GcyaDak*, p*GupCas*). After 48 h, YPH499 (p*GcyaDak*, p*GupCas*) has utilized most of glycerol provided and produced ethanol from glycerol as the carbon source with a product yield of 0.14 g per g glycerol. When compared to YPH499 (pESC-TRP), this performance represents a 3.4-fold improvement in ethanol yield. It confirms that our strategy of metabolic engineering has successfully redistributed the carbon flux to improve the production yield of ethanol.

4. Discussion

Glycerol bioconversion adds significant value to the productivity of the biodiesel industry. Throughout this paper, examples of possible biotechnological production processes based on glycerol demonstrate that this simple chemical is a promising abundant new carbon source for industrial microbiology. Glycerol can be produced either by microbial fermentation or chemical synthesis from petrochemical feedstock. Biodiesel is produced from vegetable oils and animal fats through transesterification with, for instance, ethanol or methanol (alcoholysis), generally catalyzed by NaOH or KOH and glycerol represents 10% (v/v) of the ester (Wang et al., 2001).

The metabolite concentrations of glycerol and ethanol were measured for the genetically modified *S. cerevisiae* strains during ethanol production. The YPH499 (pESC-TRP) strain was able to produce ethanol using glycerol, but yielded only 0.69 g L^{-1}. When both the *Gcy* and *Dak* genes were introduced into the YPH499 (*pGcya-*

Dak) strain, 1.66 g L^{-1} ethanol were produced. By the overexpression of the *Gcy* and *Dak* genes, ethanol production was increased up to 2.4-fold. We have also achieved high rates of ethanol production by simultaneous overexpression of *pGup1*. When both the *Gcy*, *Dak* with *Gup1* genes were introduced to the YPH499 (*pGcyaDak, pGupCas*) strain, 2.4 g L^{-1} ethanol were produced. When compared to *S. cerevisiae* WT (pESC-TRP), this performance represents a 3.4-fold improvement in ethanol yield.

Recently, fermentation of glycerol in many organisms has been reported (Gupta et al., 2009). However, glycerol uptake is frequently cited as the only example of transport mediated by facilitated diffusion across the *E. coli* inner membrane (Auer et al., 2001). Facilitated diffusion is achieved by an integral membrane protein, the glycerol facilitator *glpF* (Braun et al., 2000; Stahlberg et al., 2000). *GlpF* acts as a highly selective channel, also conducting polyalcohols and urea derivatives, for which it is stereo-selective and enantio-selective (Braun et al., 2000). In a recent paper, the production of amino acid by *Corynebacterium glutamicum* using glycerol as substrate was demonstrated (Rittmann et al., 2008). They have successfully expressed *E. coli* genes, *glpF* with *glpK* and *glpD* in *C. glutamicum* that was able to produce amino acid from glycerol.

The possibility of using glycerol to produce ethanol in *S. cerevisiae* has been previously studied, with only the pathway being described (Roberts and Hudson, 2009). It was also reported that the function of *Gup1* protects the yeast strain with interrupted glycerol production pathway from osmotic stress (Ronnow and Kielland-Brandt, 1993). Both however did not demonstrate any ethanol production. This study showed that ethanol production from glycerol is possible in principle with a recombinant *S. cerevisiae*.

Metabolic engineering has been used to broaden the carbon substrate spectrum of *S. cerevisiae*. Although glycerol is the main by-product in supernatants of cultivation of *S. cerevisiae*, to our knowledge, ethanol production by *S. cerevisiae* from glycerol has not been reported. However, the ethanol yield from glycerol fermentation described is low and future development is needed in order for this fermentation to be commercially applicable.

5. Conclusions

This work demonstrates the feasibility of converting glycerol, an inevitable by-product of biodiesel production, to ethanol by using a rational engineering approach enabled by previous studies. Engineered strains performed well when crude glycerol was used as a carbon source. Representative data for the production of ethanol are shown. The use of an industrial medium containing crude glycerol is of great relevance for the biocatalysts developed in this work. In future research, newly rational metabolic pathway alterations would help to make a profoundly increasing impact on the industrial production of ethanol.

Acknowledgements

We thank Roy H. Doi (University of California, Davis) for critical reading of the manuscript. This research was supported by the core Environmental Technology Development project for Next Generation funded by the Ministry of Environment, Republic of Korea (No. 032-091-019).

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