Biotechnol Lett (2010) 32:1077–1082
DOI 10.1007/s10529-010-0259-z

ORIGINAL RESEARCH PAPER

Enhanced production of ethanol from glycerol by engineered *Hansenula polymorpha* expressing pyruvate decarboxylase and aldehyde dehydrogenase genes from *Zymomonas mobilis*

Won-Kyung Hong · Chul-Ho Kim · Sun-Yeon Heo · Lian Hua Luo · Baek-Rock Oh · Jeong-Woo Seo

Received: 17 February 2010 / Accepted: 23 March 2010 / Published online: 31 March 2010

Abstract To improve production of ethanol from glycerol, the methylotrophic yeast *Hansenula polymorpha* was engineered to express the *pdc* and *adhB* genes encoding pyruvate decarboxylase and aldehyde dehydrogenase II from *Zymomonas mobilis*, respectively, under the control of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter. The ethanol yield was 3.3-fold higher (2.74 g l^{-1}) in the engineered yeast compared with the parent strain (0.83 g l^{-1}). Further engineering to stimulate glycerol utilization in the recombinant strain via expression of *dhaD* and *dhaKLM* genes from *Klebsiella pneumoniae* encoding glycerol dehydrogenase and dehydroxyacetone kinase, respectively, resulted in a 3.7-fold increase (3.1 g l^{-1}) in ethanol yield.

Keywords Aldehyde dehydrogenase II · Ethanol production · Glycerol · *Hansenula polymorpha* · Pyruvate decarboxylase

Electronic supplementary material The online version of this article (doi:10.1007/s10529-010-0259-z) contains supplementary material, which is available to authorized users.

W.-K. Hong · C.-H. Kim · S.-Y. Heo · L. H. Luo · B.-R. Oh · J.-W. Seo (✉)
Microbe-based Fusion Technology Research Center, Jeonbuk Branch Institute, KRIBB, Jeongeup, Jeonbuk 580-185, South Korea
e-mail: jwseo@kribb.re.kr

L. H. Luo
Institute for Molecular Biology and Genetics, Research Center of Bioactive Materials, Chonbuk National University, Jeonju, Chonbuk 561-756, South Korea

B.-R. Oh
School of Biological Science and Technology, Chonnam National University, Gwangju 500-757, South Korea

Introduction

Large amounts of raw glycerol are formed as the main by-product in biodiesel production and constitutes approx. 10% (w/w) of the total biodiesel generated (Johnson and Taconi 2007). This surplus raw glycerol has not only greatly disturbed the market for traditional glycerol in terms of price but has also created a significant environmental problem because it cannot be discharged directly into the environment without treatment (da Silva et al. 2009). Thus, considerable efforts have been directed towards development of methods to refine glycerol from a low-cost feedstock into industrially valuable materials including fuels, building blocks, and bioactive substances.

Glycerol is a non-fermentable by most microorganisms except for a group of bacteria including *Bacillus*, *Clostridium*, *Enterobacter*, *Klebsiella* and *Lactobacillus* species. Recently, Gonzalez and colleagues demonstrated

anaerobic fermentation of glycerol by *Escherichia coli* and thus offered a new metabolic platform for production of fuels and chemicals (Dharmadi et al. 2006; Gonzalez et al. 2008; Murarka et al. 2008). Moreover, the cost of ethanol production from glycerol was almost 40% lower compared with production from corn-derived sugars in terms of feedstock demand and operational costs (Yazdani and Gonzalez 2007). These observations encouraged the engineering of *E. coli* for the efficient fermentative conversion of glycerol to ethanol and co-products (Yazdani and Gonzalez 2008; Durnin et al. 2009).

The methylotrophic yeast, *Hansenula polymorpha,* is one of the most important non-conventional yeasts used in industrial processes such as heterologous protein production (Gellissen 2002). In addition, *H. polymorpha* has the potential to be useful in ethanol production owing to its ability to ferment xylose, which is derived from the hemicellulose component of biomasses (Ryabova et al. 2004; Guerra et al. 2005). In the present study, we evaluated the potential of *H. polymorpha* as a metabolic platform for conversion of glycerol to ethanol.

Materials and methods

Strains, plasmids, and media

Escherichia coli DH5α was used as the host strain for genetic cloning. *Zymomonas mobilis* ZM4 was the source of genes encoding pyruvate decarboxylase (*pdc*) and aldehyde dehydrogenase II (*aldB*). *Klebsiella pneumoniae* MGH78578 was the source of genes encoding glycerol dehydrogenase (*dhaD*) and dehydroxyacetone kinase (*dhaKLM*). The methylotrophic yeast *Hansenula polymorpha* DL1-L was used for production of ethanol from glycerol. pGEM-T-Easy (Promega) was employed for cloning and sequencing of amplified DNA fragments. pYHSA161 (Heo et al. 2003) was used to express genes in *H. polymorpha*. *E. coli* and *H. polymorpha* were grown in LB (yeast extract, 0.5%; Bacto-tryptone, 1%; and NaCl, 1%; all w/v) and YPD medium (yeast extract, 1%; peptone, 2%; glucose, 2%; all w/v) supplemented with leucine (5 g l^{-1}), respectively. Ampicillin (50 μg ml^{-1}) and G418 (0.5–2 mg ml^{-1}) were used for selection of transformants of *E. coli* and *H. polymorpha*, respectively.

Construction of plasmids pYH-pdc-adhB, pYH-dhaDKLM, and pYH-pdc-adhB-dhaDKLM

A schematic representation of the strategy used to construct pYH-pdc-adhB, pYH-dhaDKLM, and pYH-pdc-adhB-dhaDKLM is shown in Fig. 1. The primers used in amplification of DNA sequences by PCR are listed in Supplementary Table 1. The 0.8 and 0.3 kb DNA sequences, including the promoter (pGAP) upstream of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the terminator sequence (tT) of alcohol oxidase (AOX) of *H. polymorpha*, were amplified using pYHSA161 (Heo et al. 2003) as a template. Amplified DNA sequences were cloned into pGEM-T-Easy vector generating pGEM-pGAP-tT, followed by DNA sequencing to confirm the absence of any errors in the DNA polymerization. The 1.8 and 1.15 kb ORF of *pdc* and *adhB* were amplified from *Z. mobilis* chromosomal DNA using the primers as indicated in Supplementary Table 1. DNA sequences encoding *dhaD* (1.1 kb), *dhaK* (1.1 kb), *dhaL* (0.6 kb), and *dhaM* (1.4 kb) were amplified from *K. pneumoniae* chromosomal DNA using primers listed in Supplementary Table 1. Amplified DNA fragments were cloned into appropriate restriction sites of pGEM-pGAP-tT, followed by DNA sequencing to confirm the absence of any errors. To generate pYH-pdc-adhB, the *Not*I and *Spe*I–*Nhe*I fragments, including *pdc* (pGEM-pdc) and *adhB* (pGEM-adhB) were serially inserted into the *Not*I and *Spe*I sites of pYHSA161, respectively. Plasmids pYH-dhaDKLM and pYH-pdc-adhB-dhaDKLM were also similarly constructed as shown in Fig. 1.

Screening and cultivation of *H. polymorpha* transformants expressing *pdc* and *adhII* genes

Hansenula polymorpha was transformed using the lithium acetate and DMSO method (Hill et al. 1991), and the resulting Leu^+ transformants harboring pYH-pdc-adhII or pYH-pdc-adhII-dhaDKLM were stabilized as previously described (Sohn 1997).

Hansenula polymorpha strains were cultivated in 250 ml flasks with 50 ml YP medium, including glucose or glycerol as carbon source. Growth of the cells was monitored from the OD_{600} values. Cells were subjected to enzyme activity assays and the culture broth was analyzed for relevant metabolites. All results presented reflect data from three independent experiments.

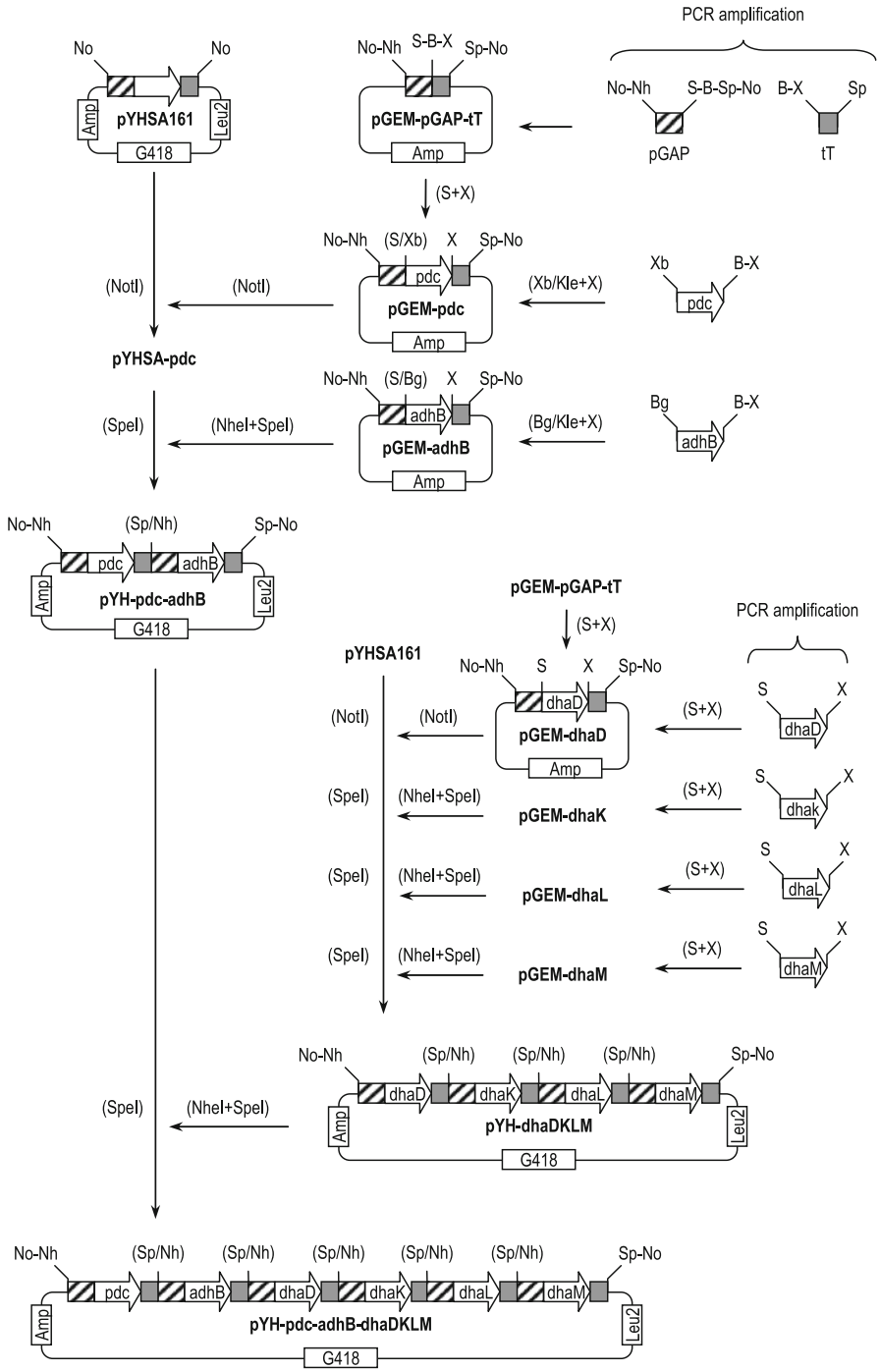

Fig. 1 Schematic representation of the strategy used for construction of pYH-pdc-adhB and pYH-pdc-adhB-dhaDKLM. Abbreviations for restriction enzymes: B: *Bam*HI, Bg: *Bgl*II, Nh: *Nhe*I, No: *Not*I, S: *Sma*I, Sp: *Spe*I, X: *Xho*I, Xb: *Xba*I. Kle is the Klenow fragment

Enzyme activity assays

The activities of pyruvate decarboxylase (Pdc) and aldehyde dehydrogenase (AdhII) were measured using the method of Postma et al. (1989), whereas the activity of glycerol dehydrogenase (DhaD) was estimated by the method of Slininger et al. (1983). One unit of enzyme activity was the amount of enzyme consuming 1 μmol substrate per min. Protein was determined using a protein assay kit (Bio-Rad), with BSA as a standard.

Metabolite analysis

The levels of residual glycerol and ethanol in culture broth were determined by HPLC equipped with a refractive index detector and an organic acid analysis column (300 × 78 mm; Aminex HPX-87H; Bio-Rad) held at 65°C. The mobile phase was 5 mM H_2SO_4 at 0.8 ml min^{-1}.

Results

Construction of a recombinant *H. polymorpha* strain transformed with *pdc* and *adhB* genes from *Z. mobilis*

Ethanol is produced in *H. polymorpha* from the reduction of pyruvate by pyruvate decarboxylase and alcohol dehydrogenase. Overexpression of the pyruvate decarboxylase gene (*PDC1*) from *H. polymorpha* or *Kluyveromyces lactis* resulted in increased production of ethanol in *H. polymorpha* using xylose as substrate (Ishcuhk et al. 2008). We investigated whether overexpression of genes encoding proteins involved in the ethanol production could also result in an increase in ethanol yield from glycerol. To this end, the recombinant plasmid pYH-pdc-adhB (Fig. 1) was prepared, in which expression of the genes *pdc* and *adhB*, encoding pyruvate decarboxylase and aldehyde dehydrogenase II from *Z. mobilis*, respectively, was driven by the constitutive *GAPDH* promoter (pGAP) and terminated by the *AOX* terminator (tAOX). Plasmid pYH-pdc-adhB was linearized and successfully transformed into *H. polymorpha* DL1-L (Leu^-). Transformants were selected by leucine prototrophy (Leu^+) on minimal medium without leucine, and stabilized by serial cultivation in such medium. To induce possible multiple integration of the relevant genes into chromosomal DNA, stabilized transformants were grown on minimal medium, without leucine, but supplemented with G418 at either 0.5, 1, or 2 mg ml^{-1}. The resulting recombinant yeast strain was termed Hp DL1-L/pYH-pdc-adhB. The presence of the required inserts in the genome of the recombinant strain was confirmed by PCR (Fig. 2).

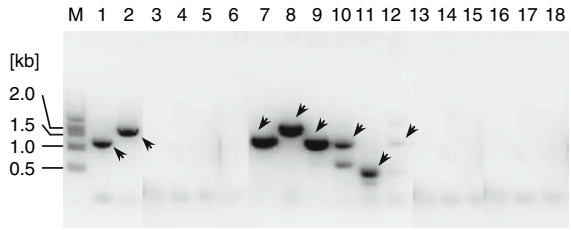

Fig. 2 Confirmation of chromosomal integration of *adhB* (lanes 1, 7, and 13; 1.15 kb), *pdc* (lanes 2, 8, and 14; 1.8 kb), *dhaD* (lanes 3, 9, and 15; 1.1 kb), *dhaK* (lanes 4, 10, and 16; 1.1 kb), *dhaL* (lanes 5, 11, and 17; 0.6 kb) and *dhaD* (lanes 6, 12, and 18; 1.4 kb) genes in recombinant *H. polymorpha* strains, as shown by PCR amplification. Lanes 1–6, HpDL1-L/pYH-pdc-adhB; lanes 7–12, HpDL1-L/pYH-pdc-adhB-dhaDKLM; lanes 13–18, HpDL1-L. M: molecular size marker

Enhanced ethanol yield from glycerol by action of the recombinant *H. polymorpha* strain Hp DL1-L/pYH-pdc-adhB

The recombinant yeast grew similarly to the control strain with glycerol at 2% (v/v) as sole carbon source (Fig. 3). The ethanol yield was 3.3-fold higher (2.74 g l^{-1}) than the parent strain (0.83 g l^{-1}), although ethanol production was still low. Consistent with these results, the activities of Pdc and AdhII were 121 and 170% higher, respectively, in Hp DL1-L/pYH-pdc-adhB than in Hp DL1-L (Table 1).

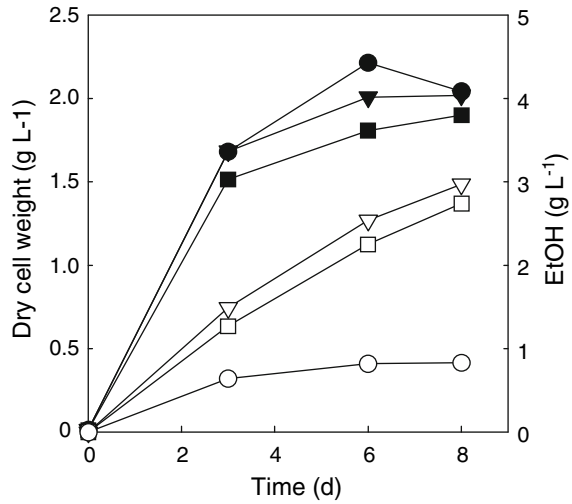

Fig. 3 Cell growth (closed symbols) and ethanol production (open symbols) of recombinant *H. polymorpha* strains. HpDL1-L, *circles*; HpDL1-L/pYH-pdc-adhB, *squares*; HpDL1-L/pYH-pdc-adhB-dhaDKLM, *triangles*

Table 1 Activities (enzyme units) of Pdc, AdhII, and DhaD in recombinant *H. polymorpha* strains

Strain	Pdc	AdhII	DhaD
H. polymorpha HpDL1-L	0.87 (100)	0.8 (100)	1.26 (100)
HpDL1-L/pYH-pdc-adhB	1.05 (121)	1.36 (170)	1.38 (110)
HpDL1-L/pYH-pdc-adhB-dhaDKLM	1.11 (128)	1.3 (163)	1.94 (154)

Proportions are shown in parentheses

Effect of overexpression of the *dhaD* and *dhaKLM* genes of *Klebsiella pneumoniae* on glycerol utilization and ethanol yield

In *K. pneumoniae*, anaerobic utilization of glycerol is catalyzed by glycerol dehydrogenase (DhaD) and dehydroxyacetone kinase (DhaKLM). To increase ethanol yield from glycerol, *dhaD* and *dhaKLM* were overexpressed in the recombinant yeast strain. The recombinant plasmid pYH-pdc-adhB-dhaDKLM was prepared by inserting *dhaDKLM* between the constitutive *GAPDH* promoter (pGAP) and the *AOX* terminator (tAOX), together with *pdc* and *adhB* (Fig. 1), and transformed into *H. polymorpha*. A transformant was selected and stabilized as described above.

Enzyme analysis showed that DhaD activity was 54 and 44% higher in Hp DL1-L/pYH-pdc-adhB-dhaDKLM than in Hp DL1-L and Hp DL1-L/pYH-pdc-adhB, respectively (Table 1). Glycerol utilization by these strains reflected the respective levels of enzyme activity (data not shown). Also, ethanol yield from glycerol was slightly increased (to 3.1 g l^{-1}) after expression of *dhaDKLM* in the recombinant yeast strain (Fig. 3).

Discussion

Ethanol production from glycerol occurs in some microbial strains, such as *Klebsiella* and *Enterobacter* species, in which the cellular redox balance during the oxidative process of glycerol was retained by a coupled reductive process producing 1,3-propanediol. The highest yield of ethanol from glycerol (17 g l^{-1}) was reported during fed-batch fermentation of *K. pneumoniae* (Cheng et al. 2007). Recently, fermentative utilization of glycerol has been reported in *E. coli* in which glycerol is usually assimilated via an aerobic metabolic pathway (Durnin et al. 2009). The production of ethanol from glycerol attained a maximum level of approximately 19 g l^{-1} in an engineered *E. coli* strain. In the present study, we constructed a recombinant *H. polymorpha* yeast strain expressing the *pdc* and *adhB* genes from *Z. mobilis*. This recombinant strain demonstrated a 3.3-fold increase in ethanol yield, from glycerol, in a flask experiment (final concentration of ethanol: 2.74 g l^{-1}).

Glycerol is dissimilated by distinct metabolic pathways in microorganisms. The relevant respiratory pathways include that involving the action of glycerol kinase (GlpK) and glyceraldehyde-3-phosphate dehydrogenase (GlpD/GlpABC) and the fermentative pathway catalyzed by glycerol dehydrogenase (DhaD) and dehydroxyacetone kinase (DhaKLM). Durnin et al. (2009) reported efficient stimulation of ethanol production from glycerol in *E. coli* by overexpression of genes involved in the fermentative pathway. Introduction of *dhaD* and *dhaKLM* from *K. pneumoniae* also resulted in an increase in glycerol utilization and ethanol yield in the recombinant yeast strain.

Although production of ethanol from glycerol increased in *H. polymorpha* after appropriate genetic engineering, the present yield is lower than that of bacteria such as *K. pneumoniae* and *E. coli*. However, we expect that a recombinant yeast strain will be useful in the development of a industrial process for ethanol production by metabolic and process engineering because a critical advantage of yeast compared with bacteria, is high-level ethanol tolerance.

Acknowledgments This research was supported by a grant from the KRIBB Research Initiative Program.

References

Cheng KK, Zhang JA, Liu DH, Sun Y, Liu HJ, Yang MD, Xu JM (2007) Pilot-scale production 1,3-propanediol using *Klebsiella pneumoniae*. Proc Biochem 42:740–744

da Silva GP, Mack M, Contiero J (2009) Glycerol: a promising and abundant carbon source for industrial microbiology. Biotechnol Adv 27:30–39

Dharmadi Y, Murarka A, Gonzalez R (2006) Anaerobic fermentation of glycerol by *Escherichia coli*: a new platform for metabolic engineering. Biotechnol Bioeng 94:821–829

Durnin G, Clomburg J, Yeates Z, Alvarez PJ, Zygourakis K, Campbell P, Gonzalez R (2009) Understanding and harnessing the microaerobic metabolism of glycerol in *Escherichia coli*. Biotechnol Bioeng 103:148–161

Gellissen G (ed) (2002) *Hansenula polymorpha*—biology and applications. Wiley-VCH, Weinheim

Gonzalez R, Murarka A, Dharmadi Y, Yazdani SS (2008) A new model for the anaerobic fermentation of glycerol in enteric bacteria: trunk and auxiliary pathways in *Escherichia coli*. Metab Eng 10:234–245

Guerra E, Chye PP, Berardi E, Piper PW (2005) Hypoxia abolishes transience of the heat-shock response in the methylotrophic yeast *Hansenula polymorpha*. Microbiology 151:805–811

Heo JH, Hong WK, Cho EY, Kim MW, Kim JY, Kim CH, Rhee SK, Kang HA (2003) Properties of the *Hansenula polymorpha*-derived constitutive *GAP* promoter, assessed using an HAS reporter gene. FEMS Yeast Res 4:175–184

Hill J, Donald KAG, Griffiths DE (1991) DMSO-enhanced whole cell yeast transformation. Nucleic Acids Res 19:5791

Ishcuhk OP, Voronovsky AY, Stasyk OV, Gayda GZ, Gonchar MV, Abbas CA, Sibirny AA (2008) Overexpression of pyruvate decarboxylase in the yeast *Hansenula polymorpha* results in increased ethanol yield in high-temperature fermentation of xylose. FEMS Yeast Res 8:1164–1174

Johnson DT, Taconi KA (2007) The glycerin glut: options for the value-added conversion of crude glycerol resulting from biodiesel production. Environ Prog 26:338–348

Murarka A, Dharmadi Y, Yazdani SS, Gonzalez R (2008) Fermentative utilization of glycerol by *Escherichia coli* and its implications for the production of fuels and chemicals. Appl Environ Microbiol 74:1124–1135

Postma E, Verduyn C, Scheffers WA, van Dijken JP (1989) Enzymatic analysis of the crabtree effect in glucose-limited chemostat cultures of *Saccharomyces cerevisiae*. Appl Environ Microbiol 55:468–477

Ryabova OB, Chmil OM, Sibirny AA (2004) Xylose and cellobiose fermentation to ethanol by the thermotolerant methylotrophic yeast *Hansenula polymorpha*. FEMS Yeast Res 4:157–164

Slininger PJ, Bothast RJ, Smiley KL (1983) Production of 3-hydroxypropionaldehyde from glycerol. Appl Environ Microbiol 46:62–67

Sohn JH (1997) Characterization of telomere-associated ARSs (autonomously replicating sequences) and their use for multiple gene integration in *Hansenula polymorpha*. Ph.D. Thesis, Korea Advanced Institute of Science and Technology, Taejon, Korea

Yazdani SS, Gonzalez R (2007) Anaerobic fermentation of glycerol: a path to economic viability for the biofuels industry. Curr Opin Biotechnol 18:213–219

Yazdani SS, Gonzalez R (2008) Engineering *Escherichia coli* for the efficient conversion of glycerol to ethanol and co-products. Metab Eng 10:340–351