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BIOENERGY/BIOFUELS/BIOCHEMICALS

The role of aldehyde/alcohol dehydrogenase (AdhE) in ethanol production from glycerol by *Klebsiella pneumoniae*

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Abstract Transcriptome analysis of a *K. pneumoniae* GEM167 mutant strain derived by irradiation with gamma rays, which exhibited high-level production of ethanol from glycerol, showed that the mutant expressed AdhE at a high level. Ethanol production decreased significantly, from 8.8 to 0.5 g l^{-1}, when an *adhE*-deficient derivative of that strain was grown on glycerol. Bacterial growth was also reduced under such conditions, showing that AdhE plays a critical role in maintenance of redox balance by catalyzing ethanol production. Overexpression of AdhE enhanced ethanol production, from pure or crude glycerol, to a maximal level of 31.9 g l^{-1} under fed-batch fermentation conditions; this is the highest level of ethanol production from glycerol reported to date.

Keywords *Klebsiella pneumoniae* · Glycerol · Ethanol · Aldehyde/alcohol dehydrogenase (AdhE)

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Introduction

The demand for biofuels is currently increasing worldwide. Optimally, renewable biomass can be used to produce such fuels. Recently, the production and use of bio ethanol and biodiesel has significantly increased. Biodiesel production generates glycerol as the principal by-product, with glycerol constituting as much as 10 % (w/w) of the biodiesel generated [10]. This great surplus of raw glycerol has negatively affected the traditional market for glycerol and has also created a significant environmental problem; glycerol cannot be discharged without further treatment [5]. Much research is being devoted to the use of glycerol as a low-cost feedstock for the creation of industrial value-added products, including fuels such as bioethanol [6].

Mu et al. [12] reported that *Klebsiella pneumoniae* produced ethanol from crude glycerol derived from biodiesel production. This strain is but one example of the many microorganisms that can ferment glycerol. The maximal concentration obtained in the cited work was 11.9 g l^{-1}, and the productivity was 0.28 g l^{-1} h^{-1}. Higher ethanol productivity was achieved by a derivative of *Klebsiella oxytoca* M5al, in which production of lactate (a competing metabolite) was reduced by deletion of the lactate dehydrogenase gene. The maximum ethanol concentration attained was 19.5 g l^{-1} and the productivity 0.56 g l^{-} h^{-1} [23]. Genetically engineered *Escherichia coli* strains can convert glycerol to ethanol under conditions of anaerobic fermentation [6, 8, 13]. An *E. coli* strain in which the gene encoding lactate dehydrogenase was deleted and that encoding aldehyde dehydrogenase overexpressed produced 20.7 g l^{-1} of ethanol from a growth medium with 60 g l^{-1} of glycerol; the productivity was 0.22 g l^{-1} h^{-1} [7]. Choi et al. [3] reported that *Kluyvera cryocrescens* S26 produced ethanol from crude glycerol, a

by-product of a palm oil-based biodiesel plant, under micro-aerobic growth conditions. The maximal level attained was 27 g l^{-1} and the productivity 0.61 g l^{-1} h^{-1}. Recently, *Pachysolen tannophilus* was used to produce ethanol, to 28.1 g l^{-1}, from crude glycerol, under staged-batch fermentation conditions [11].

Previously, using gamma-irradiation, we isolated a mutant strain of *Klebsiella pneumoniae* (termed GEM167) that showed high-level ethanol production from glycerol [15]. Upon fermentation of glycerol by this strain, the level of 1,3-propanediol production decreased dramatically (compared to the wild type), from 7.93 to 0.2 g l^{-1}, but ethanol production greatly increased, from 1.1 to 8.6 g l^{-1}. In the present study, transcriptome analysis of *K. pneumoniae* GEM167 was conducted using a microarray approach. Subsequently, the role played by aldehyde/alcohol dehydrogenase (AdhE) in ethanol production from glycerol was investigated.

Materials and methods

Bacterial strains, plasmids, and media

The *K. pneumoniae* mutant strain GEM167, derived from *K. pneumoniae* ATCC 200721, has been described previously [15]. *E. coli* DH5α was used for DNA manipulation. Microbial cells were grown in LB [yeast extract (Difco), 0.5 % (w/v); Bacto-tryptone (Difco), 1.0 % (w/v); and NaCl, 1.0 % (w/v)], or germ medium (Zhang et al., 2006), supplemented with appropriate antibiotics [ampicillin (50 μg ml^{-1}) and/or apramycin (50 μg ml^{-1})]. Germ medium contained 20 g l^{-1} glycerol, 2 g l^{-1} $(NH_4)_2SO_4$, 3.4 g l^{-1} K_2HPO_4, 1.3 g l^{-1} KH_2PO_4, 0.2 g l^{-1} $MgSO_4$, 0.02 g l^{-1} $CaCl_2$ $2H_2O$, 1 g l^{-1} yeast extract, 1 ml Fe solution [5 g l^{-1} $FeSO_4$ $7H_2O$ and 4 ml l^{-1} HCl (37 %, w/v)], and 1 ml trace element solution [70 mg l^{-1} $ZnCl_2$, 100 mg l^{-1} $MnCl_2$ $4H_2O$, 60 mg l^{-1} H_3BO_3, 200 mg l^{-1} $CoCl_2$ $4H_2O$, 20 mg l^{-1} $CuCl_2$ $2H_2O$, 25 mg l^{-1} $NiCl_2$ $6H_2O$, 35 mg l^{-1} Na_2MoO_4 $2H_2O$, and 4 ml l^{-1} HCl (37 %, w/v)]. Plasmid pGEM-T Easy (Promega, Madison, WI, USA) was employed for cloning. Plasmid pBR-*adhE* contains the *K. pneumoniae* aldehyde dehydrogenase gene.

Preparation of total RNA and cDNA for microarray analysis

The wild-type and the mutant *K. pneumoniae* GEM167 strains were cultured in glycerol containing medium (upper condition) and then the total RNA preparation was isolated with the RNeasy Mini Kits by disrupting cells and use of RNeasy spin column. Then total RNA was cleaned with the RNA Cleanup kit (Qiagen, Valencia, CA, USA). Capillary electrophoresis using an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA) was used to check the integrity of total bacterial RNA. cDNA probes for cDNA microarray analysis were prepared by reverse-transcription of total RNA (25 μg) in the presence of aminoallyl-dUTP and 6 μg of random primers (Invitrogen, Carlsbad, CA, USA) for 3 h. The cDNA probes were purified on a Microcon YM-30 column (Millipore, Bedford, MA, USA) followed by coupling to the dyes Cy3 (reference sample) or Cy5 (test sample) (Amersham Pharmacia, Uppsala, Sweden). Dye-labeled cDNA probes were purified using a QIAquick PCR Purification Kit (Qiagen). Dried dye-labeled probes were re-suspended in hybridization buffer containing 30 % (v/v) formamide, 5 × SSC, 0.1 % (w/v) SDS, and 0.1 mg ml^{-1} salmon sperm DNA. The Cy3- and Cy5-labeled cDNA probes were mixed and hybridized to a microarray slide (the "*Shigella flexneri* 2a str. 2457T plus *Shigella flexneri* 2a str. 301 virulence plasmid pCP301 Oligonucleotide 3 × 15 K microarray") (Mycroarray.com, Ann Arbor, MI, USA). After overnight incubation at 42 °C, each slide was washed twice with a solution of 2 × SSC and 0.1 % (w/v) SDS for 5 min at 42 °C, once with a solution of 0.1 × SSC and 0.1 % (w/v) SDS for 10 min at room temperature, and finally four times with 0.1 × SSC for 1 min (each wash) at room temperature. Each slide was dried by centrifugation at 3,000 ×*g* for 5 min and each hybridization image was scanned using an Axon 4000B (Axon Instruments, Union City, CA, USA).

Microarray data analysis

Each hybridization image was analyzed with the aid of GenePix Pro 3.0 software (Axon Instruments); gene expression ratios (reference vs. test) were obtained. Microarray data analysis was performed using Genowiz 4.0 (Ocimum Biosolutions, Hyderabad, India). Global lowess was used for data analysis with normalization. The cut-offs for up- and down-regulation were at a level with a linear analysis of variance (ANOVA) and these genes that are induced or repressed at greater than the 99.5 % confidence level ($p < 0.05$) in the mutant *K. pneumoniae* GEM167 versus the wild-type strain, respectively. Biologically relevant groupings yielded by microarray data were identified using both NetAffx from Affymetrix (Santa Clara, CA, USA) and the Database for Annotation, Visualization and Integrated Discovery (DAVID; version 6.7) from the National Institutes of Allergy and Infectious Diseases (Bethesda, MD, USA). The three sets of microarray data were analyzed from independent RNA preparation.

Construction of an *adhE* mutant strain

To delete the chromosomal *adhE* gene encoding aldehyde dehydrogenase, 0.65-kb DNA sequences upstream and downstream of *adhE* were amplified by PCR using oligonucleotides P1 (5′-TCCGCAGCATCATCAAAATTGGC G-3′) and P2 (5′- ACCGGAGCAACTTCGGCTTTC*GA TATC*ATTCGAGCATCTGCAGCGGC-3′; the bases in italics indicate an *Eco*RV site) binding to the upstream region and P3 (5′- GCCGCTGCAGATGCTCGAAT*GAT ATC*GAAAGCCGAAGTTGCTCCGGT-3′; bases in italics indicate an *Eco*RV site) and P4 (5′- TGTATAATCCA CAGACCTCGTTA-3′) binding to the downstream region. The PCR conditions were: Initial denaturation at 95 °C for 5 min, followed by 30 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 90 s; the final step was 72 °C for 7 min. The PCR products were annealed using primers P1 and P4 and the resultant product cloned into pGEM-T Easy. Next, an apramycin-resistance gene, *aac(3)IV*, obtained from pIJ773 by digestion with *Eco*RI and *Hin*dIII, was inserted into the *Eco*RV site of the PCR product generated above after that product was treated with the Klenow fragment. The resultant plasmid, designated pT-*adhE*-Apra, was used as a template for PCR amplification of the deletion cassette, which was next introduced into *K. pneumoniae* Δ*ldhA* by electroporation [19] prior to homologous recombination creating a chromosomal mutant. Correct integration of the DNA fragment was confirmed by Southern hybridization using regions upstream of *adhE* and *aac(3)IV* to probe *Kpn*I-digested chromosomal DNA; the probes were labeled with digoxigenin-dUTP) (Roche Diagnostics GmbH, Mannheim, Germany).

Construction of a plasmid expressing *adhE* in *K. pneumoniae*

The 2.6-kb open reading frame of *adhE* was amplified from chromosomal DNA of *K. pneumoniae* using the following primers: *adhE*-F, 5′-*TCTAGAA***ATG**GCTGTTACTAATA TCGCTGAA-3′(the bases in italics indicate an *XbaI* site and those in bold the start codon) and *adhE*-R, 5′-*GGAT CC*CTCGAGTTAAGCGGATTTTTTCGCTT-3′ (the italicized and underlined bases indicate *Bam*HI and *Xho*I sites, respectively; and the bold bases a stop codon). The *lacZ* promoter sequence (P_{lacZ}-*adhE*) was amplified using specific primers; these were P_{lacZ}-*adhE* -F (5′-*CTCGAG*TCTA GAAGCGGGCAGTGAGCGCAA-3′; the italicized and underlined base indicate *Xho*I and *Xba*I sites, respectively) and P_{lacZ}-*adhE*-R (5′-*GGATCC*AGCTGTTTCCTGTGTG AAATTG-3′; the bases in italics indicate a *Bam*HI site). Amplified DNA fragments were cloned into pGEM-T Easy and sequencing confirmed the absence of any sequence error. The *Xba*I-*Bam*HI fragment including the *adhE* gene was inserted between the equivalent restriction sites downstream of the *lacZ* promoter sequence. Next, pBR322 was cleaved with *Eco*RI, treated with the Klenow fragment, and ligated to a DNA fragment obtained by digestion of pGEM-*lacZ-adhE* with *Xho*I, followed by Klenow treatment, to yield plasmid pBR-*adhE*. The final plasmid was transformed into *K. pneumoniae* GEM 167 by electroporation.

Fermentation of *K. pneumoniae* strains

Seed cells for fermentation were prepared in 1-l flasks containing 200 ml of germ medium. Flasks were incubated at 37 °C for 12 h and cultures were subsequently inoculated into the growth vessel at a concentration of 10 % (v/v). Batch and fed-batch fermentations were conducted in a 5-l stirred-vessel system (Kobiotech Co. Ltd., Incheon, Korea) containing 2-l amounts of germ medium; all fermentation experiments were conducted at 37 °C with stirring at 200 rpm and airflow at 0.5 vvm. Unless stated otherwise, the pH was maintained at pH 7.0 ± 0.2 using 28 % (w/v) NH_4OH or 2 M HCl. Crude glycerol (80 % w/w), obtained from a local biodiesel manufacturing company (Ecosolution Co., Jeongeup, Korea), was used as the prime carbon source. All data are averages of those from three independent experiments.

Preparation of cell-free extracts for enzyme activity assays

Cells in culture were shaken overnight at 100 rpm in 250-ml flasks containing 50 ml of glycerol-based medium until the stationary phase was attained. The cells were next harvested by centrifugation (10,000 × *g*, 4 °C, 10 min). Each cell pellet was washed twice with cold potassium phosphate buffer (50 mM, pH 7.0) and suspended in cold buffer. The cells were sonicated in an ice bath for 3 s at 200 W, followed by a 5-s pause (90 cycles). Cell debris was removed by centrifugation (10,000 × *g*, 4 °C, 20 min) and enzyme activities of supernatants were measured.

Aldehyde/alcohol dehydrogenase (AdhE) activities were measured using the method of Postma et al. [17]. Alcohol dehydrogenase activity measured identically with the substitution of 10 mM acetaldehyde for acetyl-CoA in the buffer solution. One unit of enzyme activity was defined as the amount of enzyme that consumed 1 μmol of NADH per minute. Protein concentrations were determined using a protein assay kit (Bio-Rad, Hercules, CA, USA) employing BSA as standard. All activity measurements were performed in triplicate.

Metabolite analysis

The levels of residual glycerol, ethanol, 1,3-PD, acetate, lactate, succinate, and 2,3-BD were determined using an HPLC column equipped with a refractive index detector, and an organic acid analysis column (300 × 78 mm; Aminex HPX-87H; Bio-Rad). The mobile phase was 5 mM H_2SO_4 and the flow rate 0.8 ml min^{-1}. The column and cell temperatures were 65 and 45 °C, respectively. Biomass concentration was determined by measurement of optical density at 600 nm (growth OD_{600}).

Results

Microarray analysis

The microarray strategies not only will identify, isotype, and serotype pathogenic bacteria but it will also aid in the discovery of new gene functions by detecting gene expressions in different diseases and environmental conditions [1]. In this study, microarray was performed and analyzed for host and mutant samples (Supplementary Tables S1 and S2). A total of 265 genes were differentially expressed at a level with a linear analysis of variance (ANOVA) and these genes that are induced or repressed at greater than the 99.5 % confidence level ($p < 0.05$). Of these genes, 103 were up- and 162 down-regulated in the mutant *K. pneumoniae* GEM167 versus the wild-type strain, respectively (data not shown). GenPlex was used to perform hierarchical clustering analysis on three sets of microarray results (data not shown) from independent RNA preparation. Earlier, we showed that *K. pneumoniae* GEM167 expressed aldehyde dehydrogenase activity at a level threefold higher than the wild type, in line with the dramatic increase in ethanol production obtained upon fermentation by the mutant [15]. Thus, the GEM167 signature upon gene expression profiling could be defined as "high AdhE activity" with the most significant *p* value of 6.86 E−07.

Studies with the Δ*adhE* mutant strain

To examine the role played by AdhE in ethanol production, the gene was precisely deleted from *K. pneumoniae* GEM 167 via homologous recombination. Southern blotting of the parent strain and mutant strains was performed using a probe specific for the gene of interest to confirm. When a DNA fragment upstream of *adhE* was used as probe, the sizes of restriction fragments that hybridized from both the parent (2.7 kb) and mutant strains (3.8 kb) were correct. A probe derived from the apramycin-resistance gene hybridized to a fragment of equal size in the Δ*adhE* mutant, whereas no band was present in the parent strain (Fig. 1).

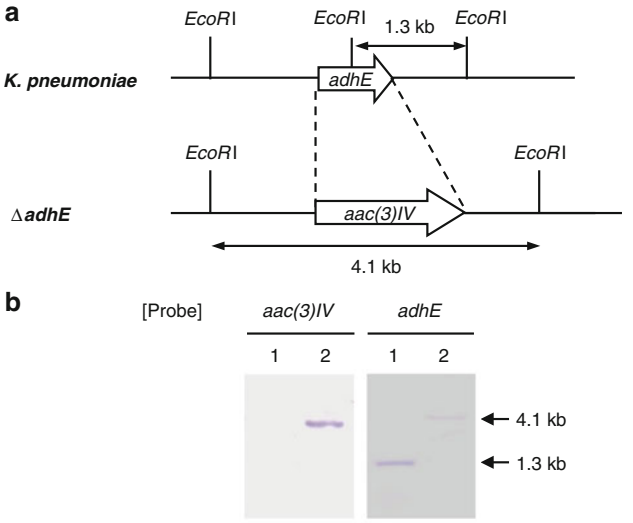

Fig. 1 Construction of an *adhE*-deficient mutant of *K. pneumoniae* (Δ*adhE*) by substitution of *adhE* with an apramycin-resistance gene [*aac(3)IV*] via homologous recombination (**a**). Correct recombination was confirmed by Southern hybridization of chromosomal DNA digested with *Eco*RI using the probes *aac(3)IV* and *adhE* (**b**). As expected, the *adhE* probe hybridized to one band (1.4 kb) in the wild-type strain and to a band of a different size (4.1 kb) in the Δ*adhE* mutant. The *aac(3)IV* probe did not react with wild-type DNA but detected the 4.1-kb band in the mutant

Table 1 Alcohol dehydrogenase activities in *K. pneumoniae* strains

Strain	Alcohol dehydrogenase activity
GEM167 Δ*ldhA*	1.77 ± 0.04
GEM167 Δ*ldhA* Δ*adhE*	0.23 ± 0.01
GEM167 Δ*ldhA* Δ*adhE*/pBR-*adhE*	1.38 ± 0.03
GEM167 Δ*ldhA*/pBR-*adhE*	2.44 ± 0.05

Enzyme activities are shown in U mg^{-1} protein

Compared with the parent strain, alcohol dehydrogenase activity was significantly decreased in the Δ*adhE* mutant (Table 1). In line with this observation, the maximum level of ethanol production and the molar yield thereof upon batch fermentation from glycerol were significantly lower in the Δ*adhE* strain; the figures were 8.8 versus 0.5 g l^{-1} and 0.88 versus 0.05 mol/mol, respectively. The levels of by-products (succinate, acetate, and 2,3-butanediol) synthesized by the mutant strain were greater than were those of the parent (Fig. 2 and Table 2). Additionally, growth of the Δ*adhE* strain was reduced during glycerol fermentation (Fig. 2), probably because the loss of AdhE activity disturbed redox flux.

Complementation of the Δ*adhE* mutation

When the *adhE* gene was re-introduced episomally, the mutant strain re-acquired enzyme activity and the ability to

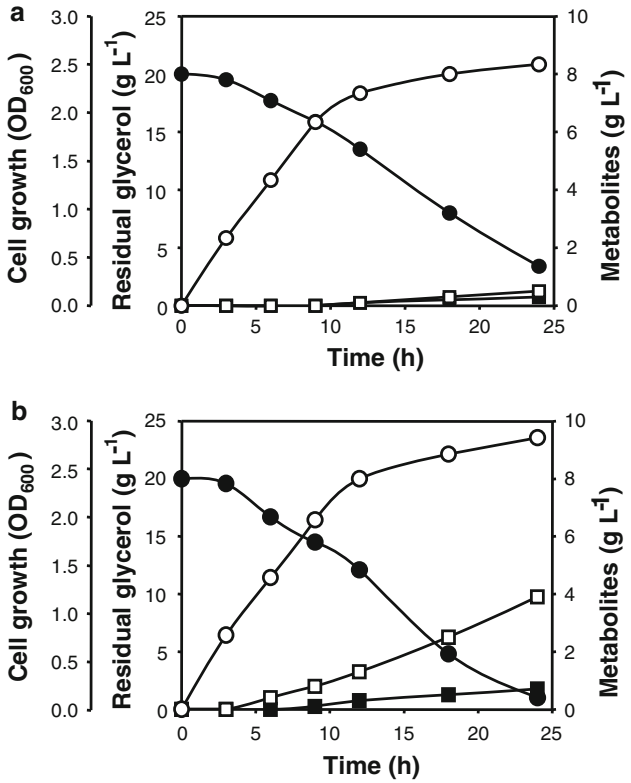

Fig. 2 Metabolites produced by of *K. pneumoniae* GEM167 Δ*ldhA* Δ*adhE* (**a**), and *K. pneumoniae* GEM167 Δ*ldhA* Δ*adhE* harboring pBR-*adhE* (**b**) upon fermentation of glycerol. *Open circles*, cell growth; *closed circles,* residual glycerol levels; *open squares*, ethanol levels; *closed squares,* acetate levels

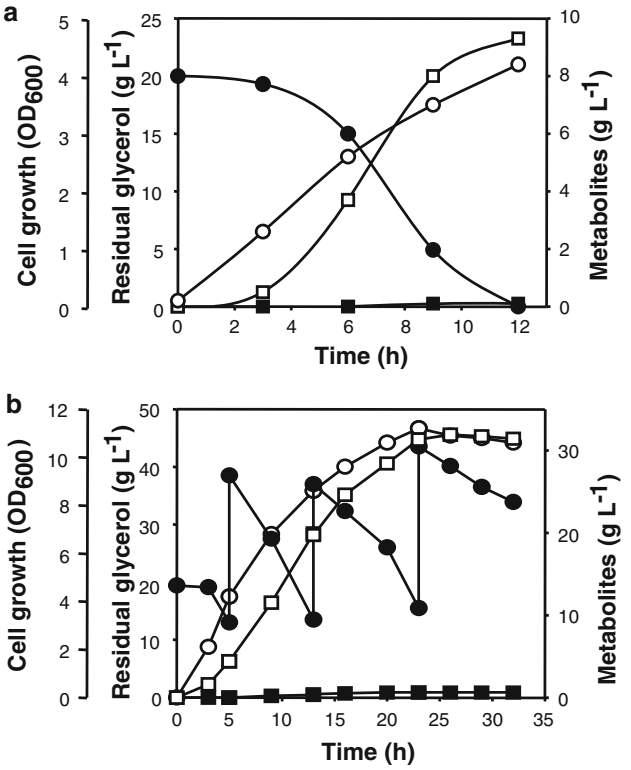

Fig. 3 Ethanol production from glycerol during batch (**a**) and fed-batch (**b**) fermentation by *K. pneumoniae* GEM167 Δ*ldhA* containing pBR-*adhE*. *Open circles*, cell growth; *closed circles*, residual glycerol levels; *open squares*, ethanol levels; *closed squares*, acetate levels

Table 2 Analysis of metabolites produced upon glycerol fermentation by *K. pneumoniae* strains

Strain	Ace	2,3-BD	EtOH	Suc	1,3-PD
GEM167 Δ*ldhA*	2.7	0.3	8.8	0.8	0.1
GEM167 Δ*ldhA* Δ*adhE*	7.5	1.0	0.5	1.3	0.3
GEM167 Δ*ldhA* Δ*adhE*/pBR-*adhE*	5.4	1.5	3.9	0.9	0.7
GEM167 Δ*ldhA*/pBR-*adhE*	3.0	0.3	9.3	0.8	0.1

All figures are g l^{-1}

Ace acetate; *Suc* succinate

produce ethanol (Table 1; Fig. 3a). Cell growth also improved but remained lower than that of the parental strain.

Enhanced production of ethanol from glycerol upon overexpression of *adhE*

As the experiments described above revealed that AdhE played a major role in production of ethanol from glycerol, we next sought to elevate ethanol production by overexpression of the *adhE* gene in a *K. pneumoniae* Δ*ldhA* mutant, in which lactate production has been rendered impossible because lactate dehydrogenase has been inactivated [15]. When *K. pneumoniae* GEM167 Δ*ldhA* harboring pBR-*adhE* was grown under batch fermentation conditions in a 5-l bioreactor (with a working volume of 2 l) at pH 7 and at 200 rpm, and with 0.5 vvm air, the level of ethanol production and the molar yield thereof were slightly increased compared to control values, from 8.8 to 9.3 g l^{-1} and from 0.88 to 0.93 mol/mol, respectively (Fig. 3a).

To further enhance ethanol production, fed-batch fermentation was performed as described in the Materials and methods section. The maximum ethanol production level, molar yield, and productivity, using pure glycerol as carbon source, were 31.9 g l^{-1}, 0.88 mol/mol, and 1.23 g l^{-1} h^{-1}, respectively (Fig. 3b). These are the highest levels reported to date. Similar values (31.1 g l^{-1}, 0.86 mol/mol, and 1.20 g l^{-1} h^{-1}) were obtained using crude glycerol derived from the biodiesel industry as a carbon source (data not shown). After fed-batch fermentation for 36 h (which equates to approximately 110 generations), the plasmid was maintained by 97 % of cells (data not shown).

Table 3 Comparison of ethanol production from glycerol by different microorganisms

Organism	Fermentation method	Ethanol production		Reference
		Concentration (g l^{-1})	Productivity (g l^{-1} h^{-1})	
Kluvvera cryocrescens S26	Batch	27	0.61	[3]
Escherichia coli EH05	Batch	20.7	0.22	[7]
Pachysolen tannophilus CBS4044	Batch	28.1	0.06	[11]
Klebsiella oxytoca M5al	Fed-batch	19.5	0.56	[23]
Klebsiella pneumoniae DSM 2026	Fed-batch	11.9	0.28	[12]
K. pneumoniae GEM167	Fed-batch	21.5	0.93	[15]
K. pneumoniae GEM167/pBR-*pdc-adh*	Fed-batch	25.0	0.78	[15]
K. pneumoniae GEM167Δ*ldhA*/pBR-*adhE*	Fed-batch	31.9	1.23	Present study

Discussion

Klebsiella pneumoniae is a well-known glycerol-fermenting microorganism. Recently, we showed that a mutant strain of *K. pneumoniae* created by gamma-ray irradiation, termed GEM167, showed remarkable enhancement of the ability to produce ethanol from glycerol [15]. In the present study, transcriptome analysis, using a microarray assay, was performed to compare the gene expression profiles of GEM167 and the *K. pneumoniae* MGH 78578 wild-type strain. A total of 265 genes were differentially expressed, 103 were up- and 162 down-regulated in the mutant *K. pneumoniae* GEM167 versus the wild-type strain, respectively (data not shown) with under *p* value cut-off 0.05. The differentially expressed genes were categorized into 19 functional groups on the basis of the principal functions of the genes. The gene expression profile of GEM167 was distinct; genes involved in energy production and conversion, coenzyme metabolism, amino acid transport and metabolism, and oxidation. NAD-dependent *adhE* showed the highest level of up-regulation, followed by genes involved in the biosynthesis of secondary metabolites, transport and catabolism, and glycerolipid metabolism. Notably, expression of the *adhE* gene, encoding *adhE*, showed the most significant *p* value of 6.86E-07. To explore the role played by *adhE*, the gene was deleted from GEM167, and the effect thereof on ethanol production from glycerol was examined. The Δ*adhE* strain failed to produce ethanol as a fermentation product, indicating that AdhE played a major role in ethanol production. AdhE activity in this context is also important in *E. coli*, *K. oxytoca*, and *Thermoanaerobacter mathranii* [9, 16, 18, 21, 22, 24]. The present report is the first to describe the role played by AdhE in glycerol fermentation by *K. pneumoniae*.

Upon glycerol fermentation by *K. pneumoniae* under either anaerobic or micro-aerobic conditions, NADH-dependent 1,3-propanediol oxidoreductase (DhaT), catalyzing 1,3-propanediol synthesis, plays a key role in the maintenance of the intracellular redox balance. NADH-dependent AdhE activity was elevated in a mutant *K. pneumoniae* GEM167 defective in DhaT activity [15]; this may have occurred in an effort to counter the probable redox imbalance caused by the DhaT defect. Consistent with this notion, growth of the Δ*adhE* derivative of GEM167 was reduced during glycerol fermentation. Such poor growth of *adhE*-deficient strains under anaerobic conditions was also evident in work with strains of *E. coli* and *Giardia lamblia* [4, 20].

Enhanced production of ethanol has been reported upon overexpression of *adhE* in other microbes including *E. coli*. For example, Nikel et al. [14] found that overexpression of *adhE* from *Leuconostoc mesenteroides* in *E. coli* CT1062 caused a 1.4-fold increase in ethanol production. A similar degree of enhancement (from 49.4 to 57 mM) was also evident in work with *Thermoanaerobacter mathranii* [22]. In *K. pneumoniae* GEM167 Δ*ldhA* harboring pBR-*adhE*, the yield of ethanol from glycerol upon batch fermentation increased from 8.8 to 9.3 g l^{-1}. Upon fed-batch fermentation using pure or crude glycerol as carbon source, the maximum levels of ethanol production increased to 31.9 and 31.1 g l^{-1} respectively; these are the highest levels of ethanol production from glycerol reported to date. In addition, the ethanol productivity (1.23 g l^{-1} h^{-1}) of our recombinant strain is the best noted to date (Table 3).

The ethanol production level should be at least 40 g l^{-1} [about 5 % (v/v)] before downstream distillation becomes economic. However, *K. pneumoniae* did not grow in glycerol-containing medium with more than 40 g L^{-1} ethanol (data not shown). Therefore, the ethanol tolerance of *K. pneumoniae* must be improved. Recently, Brown et al. [2] identified a mutant strain *Clostridium thermocellum* acquiring ethanol tolerance, in which the *adhE* gene was affected.

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