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Improved bioethanol production from metabolic engineering of *Enterobacter aerogenes* ATCC 29007

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ABSTRACT

This study investigates the enhancement of bioethanol production using a genetic engineering approach. The bioethanol-producing strain, *E. aerogenes* ATCC 29007, was engineered by deleting the D-*lactate dehydrogenase* (*ldh*A) gene to block the production of lactic acid. The Open-reading frame coding region of *ldh*A gene was replaced with a kanamycin cassette flanked by FLP recognition target sites by using a one-step method to inactivate chromosomal genes and primers designed to create in-frame deletions upon excision of the resistance cassette. The colony PCR was used to confirm the deleted gene. Glycerol, a useful byproduct in the biodiesel industry, was employed to convert into bioethanol, using engineered *E. aerogenes* SUMI014. Under optimal conditions of fermentation (34 °C, pH 7.5, 78 h), bioethanol production by the mutant strain was 34.54 g/L, 1.5 times greater than that produced by its wild type (13.09 g/L). Subsequent overexpression of *alcohol dehydrogenase* (*adh*E) gene in the mutant strain; increased the production of bioethanol up to 38.32 g/L. By the combination of gene deletion and overexpression, the bioethanol yield was 0.48 g/g when employing 80 g/L glycerol. Hence, a significant enhancement in ethanol production was observed. These results may provide valuable guidelines for further engineering bioethanol producers.

1. Introduction

Glycerol has become an inexpensive and abundant carbon source due to its inevitable generation as a by-product of biodiesel fuel production. With every 100 lbs of biodiesel produced by transesterification of vegetable oils or animal fats, 10 lbs of crude glycerol is generated [1]. The tremendous growth of the biodiesel industry created a glycerol surplus that resulted in a dramatic 10-fold decrease in crude glycerol prices over the last 2 years [1]. This decrease represents a problem for industries producing and refining glycerol, greatly affecting the economic viability of the biodiesel industry itself [1–4]. Glycerol can be used as a carbon source by a number of microorganisms, and can be converted into various interesting products such as 1, 3-propanediol, succinic acid, dihydroxyacetone, and ethanol [4]. Thus, there is an urgent need for the development of a practical process for converting glycerol into useful product.

Currently, many research groups are studying the utilization of glycerol as a carbon source for the transformation of other products such as ethanol [5] and amino acids [6]. Biodiesel is mostly produced by reacting a fat or oil (triglycerides) with methanol or ethanol in the presence of an alkali catalyst [7]. Although crude glycerol can be used as boiler fuel and as a supplement for animal feed, the market value of crude glycerol is still very low [8].

Bioethanol is a combustible fuel that can be made using well-known fermentation technology from a wide range of carbohydrate feedstock [9,10]. High ethanol yield is becoming increasingly important in order to enhance the economic viability of the commercial process. This is likely to require a combination of both strain development and improved process technology. Industrial production of ethanol from carbohydrate feedstock, such as glycerol, requires that the producing organisms not only tolerate and produce high levels of ethanol but also be able to convert the substrate directly to the end-product [11,12]. Ethanol has a positive environmental impact because of the low levels of pollution resulting from its combustion [13]. A great deal of research has been directed towards economical ethanol generation [14–16].

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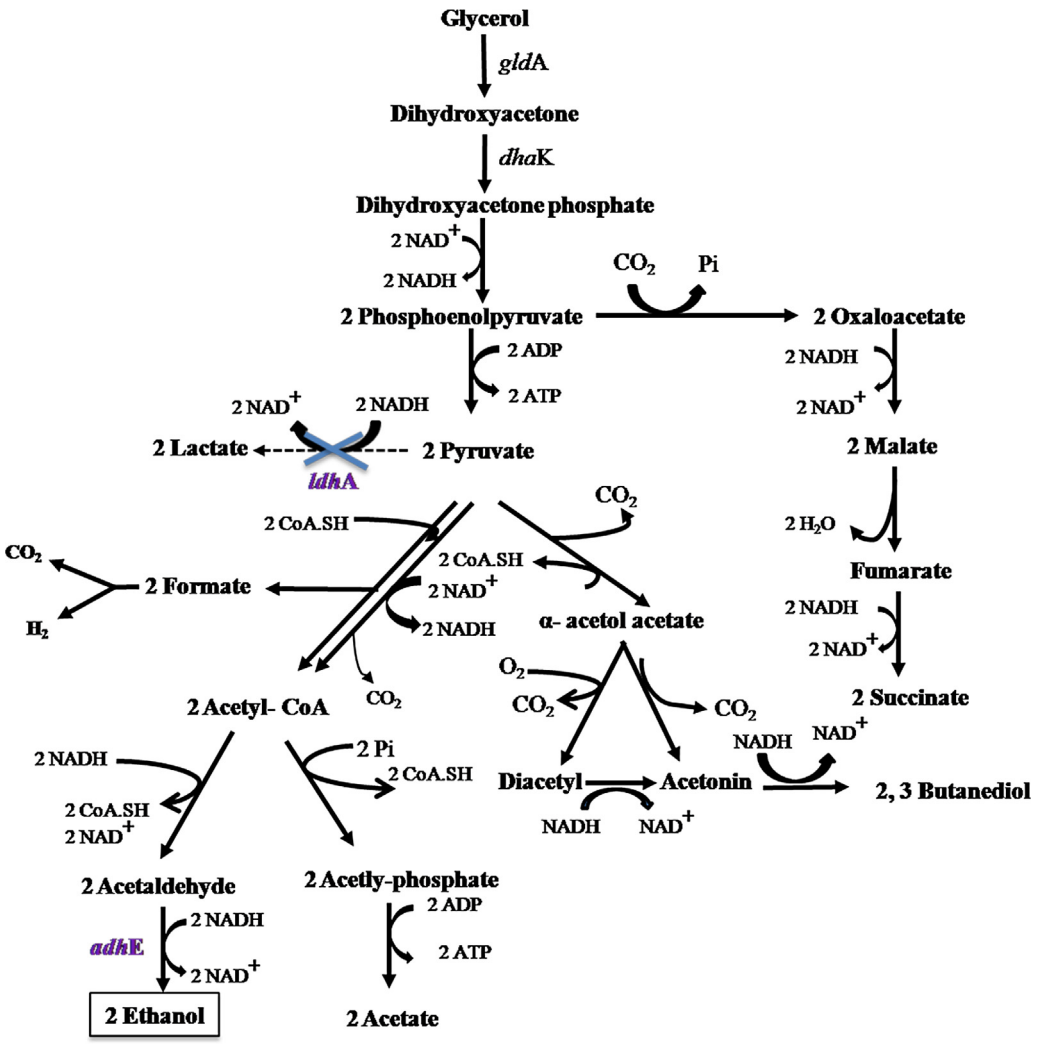

Fig. 1. Fermentative pathway of glycerol in *E. aerogenes* ATCC 29007 for the production of bioethanol and byproducts in aerobic conditions. Where, *ldh*A is, D-*lactate dehydrogenase*, and *adh*E is, *alcohol dehydrogenase* genes.

Nowadays many groups are using microorganisms for the production of bioethanol using glycerol as a carbon source. *Saccharomyces cerevisiae* is the preferred bioethanol production host, primarily as a result of being generally recognized as safe, having a proven industrial process robustness, and demonstrating good physiological and genetic characterization [17]. There are a number of other candidate ethanol-producing microorganisms, e.g. *Pichia stipitis*, *Pachysolen tannophilus*, *Klebsiella oxytoca*, *Erwinia chrysantemi*, *Lactobacillus*, *Escherichia coli, Zymomonas mobilis* and Clostridium species, among many others [18,19], but none of them have all of the desirable traits of an ideal ethanologen [20]. *E. aerogenes* can rapidly assimilate carbon sources, such as glucose, mannitol and glycerol, under pH ranges of 6-8, and it effectively produces biofuels, such as 2,3-butanediol, hydrogen, and ethanol, under anaerobic and aerobic conditions [21–25]. Recently, the entire genome sequence of *E. aerogenes* KCTC 2190 was determined [26]. Thus, the aerobic and anaerobic central pathways involved in ethanol, lactate, 2,3-butanediol, and succinate formation can be predicted. The availability of this information provided an incentive to evaluate the suitability of this organism as a platform for ethanol production under aerobic and anaerobic conditions.

E. aerogenes ATCC 29007 is a gram-negative microorganism. It can grow very well in both aerobic and anaerobic conditions [25]. The highest conversion rate of pure glycerol into ethanol has been described by Nwachukwu et al. [23,24]; *E. aerogenes* ATCC 13048 converted 18.5 g/L P-glycerol and 17.8 g/L R-glycerol into 12 and 12.8 g/L ethanol, respectively. This level is lower than that produced by *E. aerogenes* ATCC 29007 [25]. *E. aerogenes* ATCC 29007 can also grow very well and produce a greater amount of bioethanol than that produced by *E. aerogenes* KCTC 2190, of which the genome has already been sequenced. Therefore, the increase in cell growth and bioethanol production suggests that *E. aerogenes* ATCC 29007 is a better strain for bioethanol production by metabolic engineering.

The main aim of this study was to improve the production of bioethanol through the metabolic engineering of *E. aerogenes* ATCC 29007. In our previous works [25,27], we described the utilization of glycerol for the production of bioethanol by wild type *E. aerogenes* ATCC 29007, but the conversion rate was low. Therefore, in this study, we developed genetically engineered strain *E. aerogenes* SUMI014 by deleting *ldh*A gene. Lactate being the major byproduct during bioethanol fermentation makes the growth media acidic thereby reducing the cell growth. Hence, the deletion of the lactate producing gene favors cell growth and increases in ADH activity. Similarly, *adh*E gene was overexpressed to generate *E. aerogene* SUMI2008 strain to improve the *alcohol dehydrogenase* activity of *E. aerogenes* (Fig. 1). Cell growth and bioethanol production by engineered strains were increased 1.5 fold as compared with those of the wild type when utilizing glycerol as a carbon source.

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Table 1
Strains, plasmids and primers used in this study.

Strains, plasmids and primers	Genotype, relevant characteristics or sequence	Source or reference
Strains		
E. aerogenes ATCC 29007	Wild type	American type culture collection
E. aerogenes SUMI014	*ldh*A deleted mutant of*E. aerogenes* ATCC 29007	This study
E. aerogenes SUMI2008	*adh*Eoverexpressed *E. aerogenes* SUMI014	This study
Plasmids		
pKM208	LacI, λ Red + Gam-producing vector, Amp^r	Addgene
706-FLP	FLP recombinase producing plasmid, Tet^r	Gene bridges
pGEX-4T-3	Expression plasmid, Amp^r	Addegne
pGEXLPT	Recombinant plasmid containing *adh*E gene	This study
Primers		
*ldh*AF	GGAAAGCCTTATGAAAATCG	This study
*ldh*AR	GGAAAGAAGCTTAGACCAG	This study
*ldh*ADF	TGACCGAAAAGACCGCGAAAACGGCCAACGGCTGT GAAGCGGTAAATTAACCCTCACTAAAGGGCGG	This study
ldhADR	TGGTGCCCGGTAAACAGGACGTTGTGGCAGGCGGG AGAGCCGGCTACGACTCACTATAGGGCTCG	This study
*adh*EF	CGAGCTCTGTTACTGAAGCGATGAATA	This study
*adh*ER	CGAAGCTTAGTTTTTAGGATTACATC	This study

2. Materials and methods

2.1. Microorganisms, plasmids and culture condition

In this study, the ethanol-producing strain of *Enterobacter aerogenes* ATCC 29007 was used. All of the resulting strains and plasmids used in their construction are listed in Table 1. The strain was grown at 37 °C and maintained on LB-agar plates containing 10 g/L peptone, 5 g/L yeast extract and 5 g/L NaCl. The cultures were maintained at 4 °C and subcultured at regular intervals of 15 days.

2.2. Metabolic engineering

A chromosomal gene was targeted for mutation with PCR products containing a kanamycin resistance cassette flanked by FLP recognition target (FRT) sites (Fig. 2A). The *E. aerogenes* ATCC29007 D-*lactate gehydrogenase* (*ldh*A) gene of 990 bps in size was amplified by using degenerate primers *ldh*AF and *ldh*AR. Then the PCR product was sequenced by Macrogen Inc. (Seoul, South Korea). The *E. aerogense* KCTC 2190 and *Klebsiella pneumonia ldh*A homolog was identified using BLAST and deposited in GenBank (Accession no. KP296790). The disruption primers *ldh*ADF and *ldh*ADR were designed to delete 700 bps of the *ldh*A gene (Table 1). Then a disruption kanamycin cassette of 1.7 kb in size was constructed using PCR on a thermal cycler with FRT-PGK-gb2-neo-FRT as the template (Gene Bridge, Cat. No. K006). This cassette contains a prokaryotic promoter (gb2) for expression of kanamycin resistance in bacteria with a eukaryotic promoter (PGK) for the expression of neomycin resistance in mammalian cells. It also contains FRT or loxP sites inserted by Red/ET recombination of FRT or loxP flanked functional cassettes into any designated locus with subsequent removal of the selection marker by FLPe or core recombinases. Thereafter, the pKM208 plasmid was transformed into *E. aerogenes* ATCC29007 grown in Luria Broth (LB) media using an electroporator at 1700 V and transformants were selected on LB-agar plates containing 75 μg/mL carbenicilln at 30 °C. The pKM208 plasmid contains the λ red and gam genes under the tac promoter [28]. Due to its temperature sensitive origin of replication, this plasmid must be grown at 30 °C. *E. aerogenes* ATCC 29007 containing pKM208 was cultured at 30 °C, and isopropyl β-D-1-thiogalactopyranoside (IPTG) was added at a final concentration of 1 mM until OD_{600} reached 0.2. The D-*lactate dehydrogenase* (*ldh*A) knockout mutant, *Enterobacter aerogenes* SUMI014, was generated using the λ-Red recombination method (Gene Bridge, Cat. No. K006). The linear PCR product of the kanamycin resistance cassette was transformed using electroporation when OD_{600} reached 0.6. The transformants were selected at 37 °C on LB-agar plates containing 50 μg/mL kanamycin. Deletion of the *ldh*A homolog was confirmed by PCR using the *ldh*AF and *ldh*AR primer sets (Fig. 2B).

The 706-FLP plasmid was transformed into the mutant strain *E. aerogenes* SUMI014 to remove the kanamycin resistance gene by FRT recombination. This plasmid carries a pSC101 origin of replication and will maintain low copy and replicate at 30 °C. It will not propagate and will lose when incubated at 37 °C. The expression of the FLP-recombinases is driven by the thermo-sensitive promoter cI578 (λPR promoter). Therefore, expression is repressed at 30 °C and when incubated at 37 °C. The next day a single colony was picked and incubated in 1 mL LB medium without antibiotics at 30 °C for 2–3 h. Then, the temperature was changed to 37 °C, and the colony was incubated overnight. The sample was cultured on an LB agar plate and incubated at 37 °C. The next day, one colony was analyzed by PCR for the successful removal of the kanamycin cassette (Fig 2B). These colonies were incubated on LB agar plates in parallel, one condition containing 50 μg/L kanamycin and other one without kanamycin antibiotics. Colonies were successfully grown only on LB agar plates without kanamycin but no colonies grew on LB agar kanamycin plates. The colonies were transformed to LB plates containing 3 μg/mL tetracycline and incubated at 37 °C. The growth of colonies on LB agar plates without kanamycin but not on kanamycin and tetracycline LB agar plates confirmed the successful removal of the kanamycin resistance gene.

2.3. DNA manipulation

The standard protocols were applied for the isolation of DNA and manipulation of genetic material, using procedures such as deoxyribonucleic acid (DNA) isolation, restriction endonuclease digestion, and DNA ligation in *E. coli* [29,30]. Restriction enzymes and T4 DNA ligase were purchased from Roche (Germany) and iNtRON biotechnology (Korea). An agarose gel extraction kit (MEGAquick-spin™ Total Fragment DNA Purification kit, iNtRON Biotechnology, Inc.) was used to isolate the large-scale plasmid DNA from the gel.

2.4. Fermentation medium and culture conditions

The medium constituents for the fermentation of engineered *E. aerogenes* SUMI014 were 2 g/L $(NH_4)_2$ SO_4, 1.8 g/L $MgSO_4$, 7 g/L KH_2PO_4, 7 g/L K_2HPO_4, 5 g/L peptone, 3 g/L yeast extract, 3 g/L NaCl, 0.002 g/L $FeSO_4$, 0.002 g/L $ZnSO_4$ and 0.002 g/L $MnSO_4$ used as a ref-

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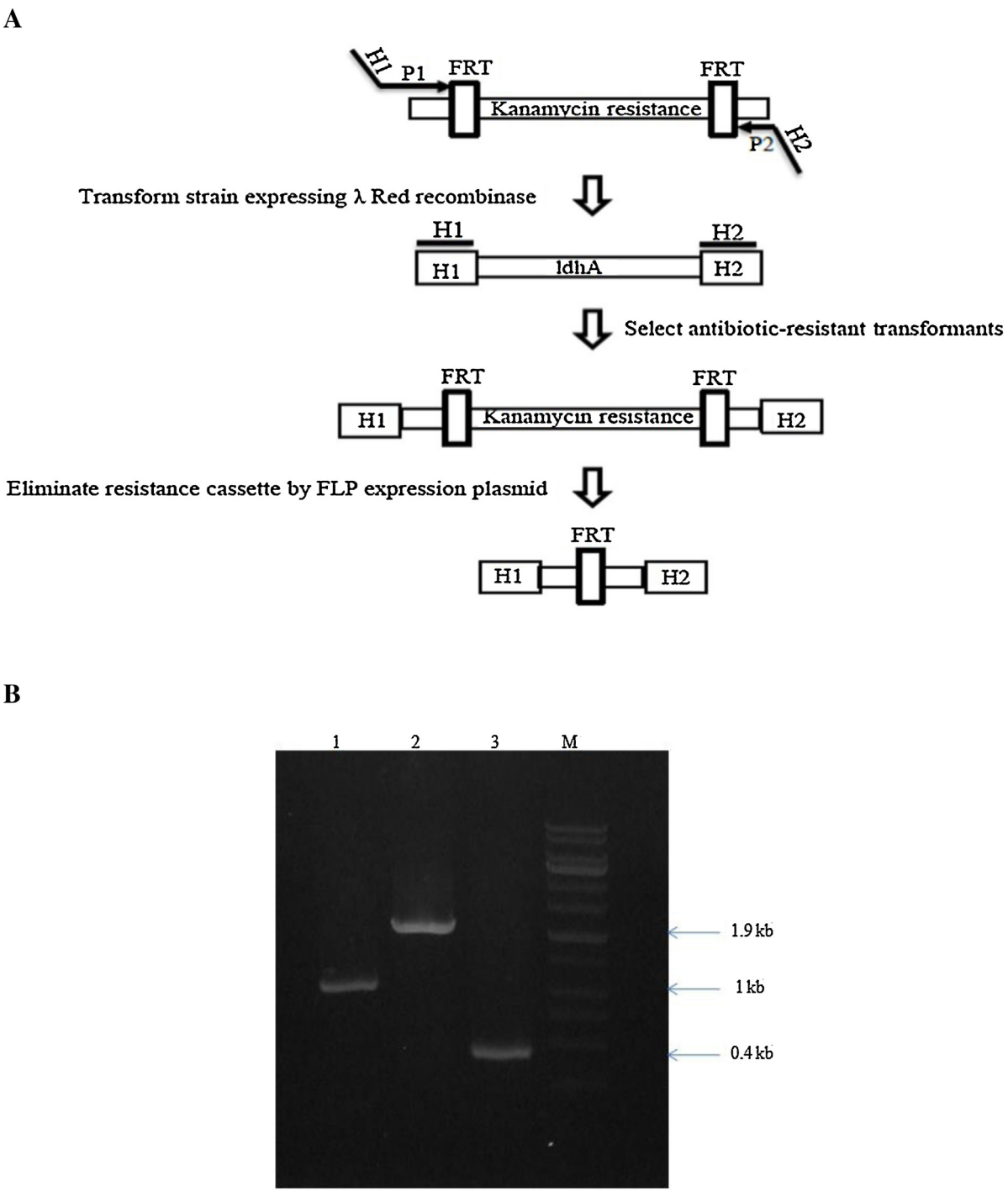

Fig. 2. Primer design and construction of a single-gene deletion mutant. Gene knockout primers have 20-nt ends for priming upstream (P1) and downstream (P2) of the FRT sites flanking the kanamycin resistance gene, and 44-nt ends of homologous (H1) gene for priming upstream (H1) and downstream (H2) of the *ldh*A gene (A). Agarose gel picture for confirmation of deletion of *ldh*A (D-*lactate dehydrogenase*) gene by colony PCR (B). Where, (1) is wild type, (2) is confirmation of *ldh*A gene deletion, (3) is confirmation of removal of kanamycin cassette and and (M) is marker.

erence [25]. Initially 60 g/L glycerol was used as a carbon source. The pH of the production medium was adjusted to 7 before autoclaving at 121 °C for 15 min. To investigate the production of bioethanol in anaerobic conditions, the production medium was degassed by sealing 50 mL of the medium in a 100 mL serum bottle by using a butyl rubber stopper, and purging with aseptic N_2 (99.99 % purity) for 2 min. The bacterial cells were cultivated at 37 °C in a 250 mL Erlenmeyer flask containing 50 mL of LB-medium at 200 rpm. When the cell culture reached an OD_{600} value of 0.2, 1 mL of the seed culture was transferred into a 100 mL serum bottle and incubated at 34 °C for 48 h. Similarly, the aerobic cultivation was carried out at 34 °C in a 250 mL Erlenmeyer flask containing 100 mL production media for 48 h at 200 rpm. Each experiment was performed in duplicate, and average was used to generate final result.

2.5. Effect of temperature, time profile and initial pH of the medium

The effects of physical parameters such as time, temperature and initial media pH, were studied for the fermentation of *E.*

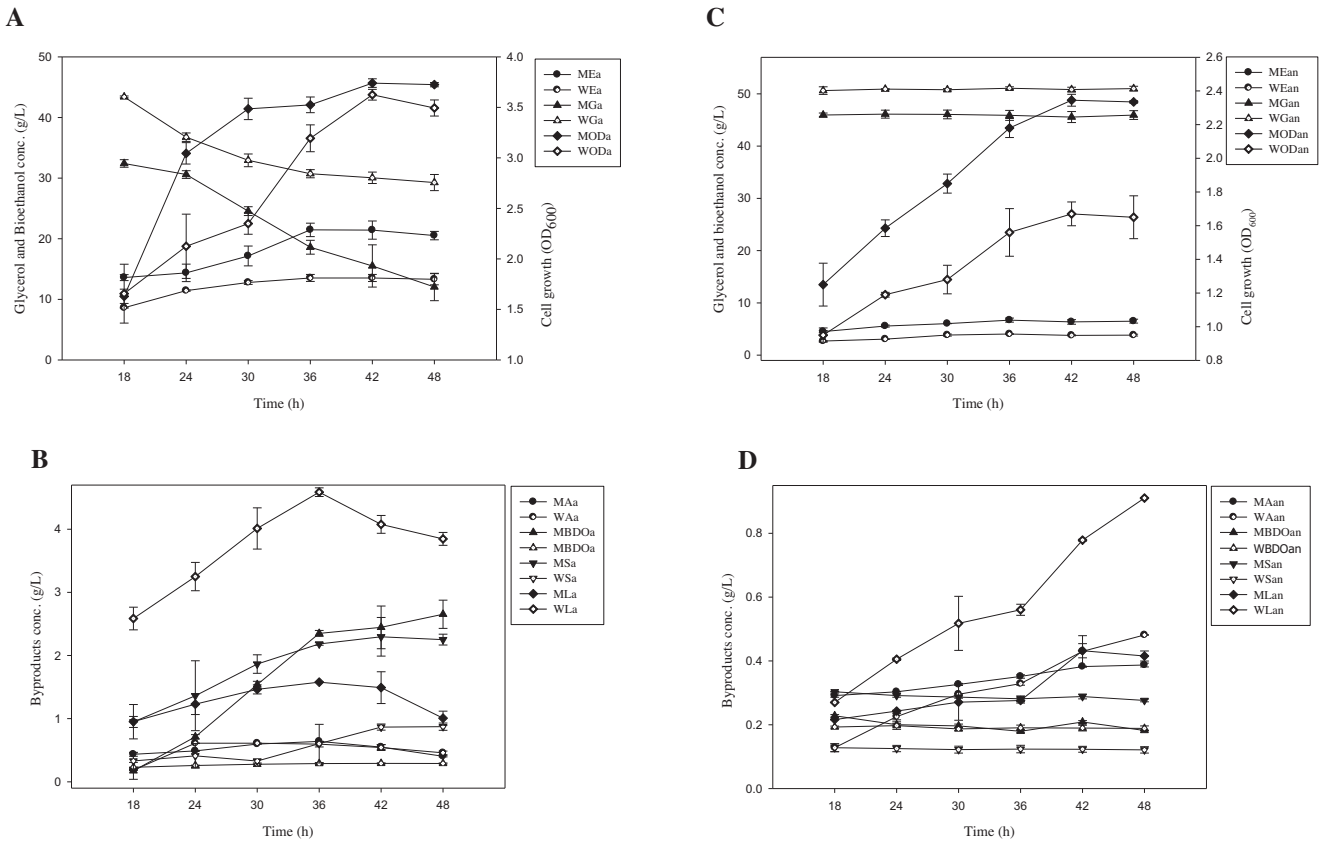

Fig. 3. Production of (A) bioethanol in aerobic conditions, (B) byproducts in aerobic conditions, (C) bioethanol in anaerobic conditions, and (D) byproducts in anaerobic conditions by *E. aerogenes* ATCC 29007 and *E. aerogenes* SUMI014. Where, ME = is ethanol produced by the mutant strain, WE = is ethanol produced by the wild strain, MG = is glycerol consumption rate by the mutant, WG = is glycerol consumption rate by the wild type strain, MOD = is cell growth rate of the mutant, WOD = is cell growth rate of the wild type strain, MA = is acetate produced by the mutant strain, WA = is acetate produced by the wild type, MBDO = is 2,3-butanediol produced by the mutant strain, WBDO = 2,3-butanediol produced by the wild, MS = is succinate produced by the mutant strain, WS = succinate produced by the wild type, ML = is lactate produced by the mutant strain, WL = lactate produced by the wild strain, a = is aerobic and, an = is anaerobic.

aerogenes SUMI014. The effects of temperature on bioethanol production were verified by incubating *E. aerogenes* SUMI014 at 32, 34, and 37 °C for various durations of 15–48 h at pH 7 and 200 rpm (Fig. 4A). Similarly, the effects of pH on bioethanol production were tested by adjusting production medium pH to 4, 4.5, 5, 5.5, 6, 6.5, 7.0, 7.5, 8, and 8.5 before autoclaving (Fig. 4B). These experiments were investigated at 34 °C and 200 rpm for 48 h.

2.6. Effect of phosphate salts

The effect of potassium phosphate (K_2HPO_4 and KH_2PO_4) salts on the production of bioethanol by mutant strain *E. aerogenese* SUMI014 was tested at 34 °C by adding same amount of both salts in the range of 4.5–7.5 g/L at pH 7.5 for 48 h (Fig. 5).

2.7. Overexpression of adh*E* gene

The *alcohol dehydrogenase* (*adh*E) gene was amplified by PCR from chromosomal DNA of *E. aerogenes* KCTC 2190 with primers *adh*E-F and *adh*E-R (Table 1), followed by cloning into the pGEX-4T-3 plasmid using *Bam*HI/*Eco*RI restriction enzymes, to yield pGEXLPT (Fig. 6A). The recombinant plasmid, pGEXLPT, was then transformed into mutant strain *E. aerogenes* SUMI014 through electroporation. Cells were plated on LB-agar plates containing 75 μg/mL carbenicilln and incubated at 37 °C for 12 h to select positive transformant *E. aerogenes* SUMI2008. The recombinant strain *E. aerogenes* SUMI2008 was cultured in LB liquid medium with 75 μg/mL carbenicilln and plasmid DNA was prepared. Then, the transformation of recombinant plasmid pGEXLPT was confirmed by *Bam*H/*Eco*RI restriction enzymes digestion (data not shown). Similarly, the empty vector pGEX-3Z-4 was also transformed into mutant strain *E. aerogenes* SUMI014, yielding a plasmid-control strain.

2.8. Alcohol dehydrogenase assay

The enzyme activities of *alcohol dehydrogenase* (ADH) in mutant strain *E. aerogenes* SUMI014, recombinant mutant strain *E. aerogenes* SUMI2008, control mutant strain *E. aerogenes* SUMI014 (with empty plasmid), and parent strain *E. aerogenes* ATCC 29007 were determined according to the method described by BioViosion [catalog K787-100]. To determine the enzyme activity, cells were harvested after 42 h of culture under aerobic conditions; cells were homogenized in 200 μl ice cold assay buffer (catalog K787-100-1), and then centrifuged at 4 °C to remove insoluble materials. The ADH reaction mixtures contained the following in a total volume of 150 μL: 50 μL of crude extracts, 8 μL developer, 10 μL of substrate (isopropanol), and 82 μL ADH assay buffer. The reaction mixture was incubated for 30 min at 37 °C, and then OD was measured in a UV–vis spectrophotometer at 450 nm to calculate the *alcohol dehydrogenase* activity (Table 2).

2.9. Analytical methods

Similarly, a UV–vis spectrophotometer (Biomate 5) was used to measure the optical density (OD) at 600 nm in order to monitor

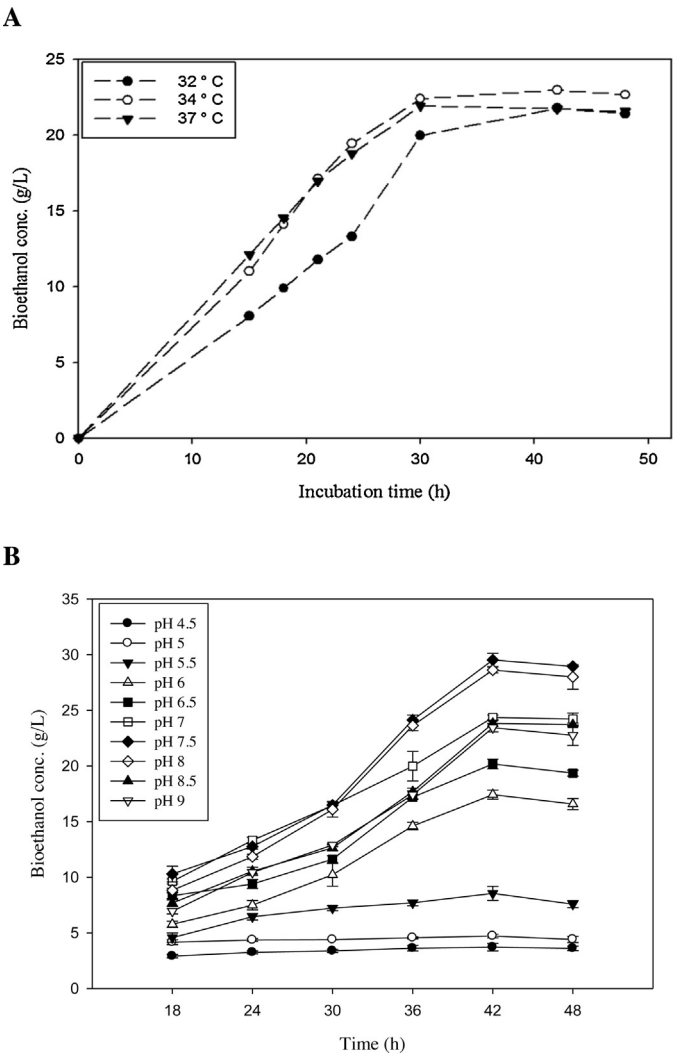

Fig. 4. Effect of (A) temperature and incubation time at pH 7, (B) pH at 34 °C on the production of bioethanol by mutant *E. aerogenes* SUMI014 at different fermentation period.

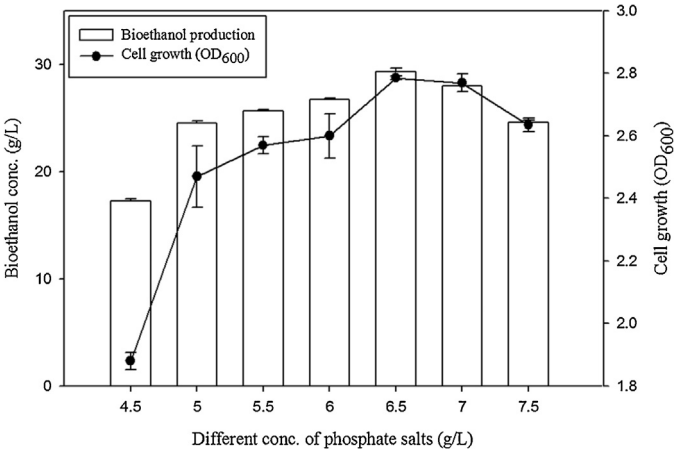

Fig. 5. Effect of phosphate salts on the production of bioethanol by mutant strain *E. aerogenes* SUMI014.

Table 2
Alcohol dehydrogenase (ADH) activity of *E. aerogenes* ATCC 29007, *E. aerogenes* SUMI014, *E. aerogenes* SUMI2008, and the control *E. aerogenes* SUMI014.

Strains	ADH activity (U/mg)
E. aerogenes ATCC 29007	56.48 ± 4.00
E. aerogenes SUMI014	234.15 ± 7.58
E. aerogenes SUMI2008	293.64 ± 1.11
E. aerogenes SUMI 014 with empty plasmid	166.37 ± 4.47

cell growth. The analysis of bioethanol and the byproducts obtained from wild type *E. aerogenes* ATCC 2900, *E. aerogenes* SUMI014, and *E. aerogenes* SUMI2008 was carried out by high-performance liquid chromatography (HPLC) using an Aminex HPX-87H column (300 9 7.8 mm, Bio-Rad, Hercules, USA) and a refractive index detector (RID-10A, Shimadzu, Tokyo, Japan). The temperature of the column and detector was maintained at 50 °C. The mobile phase was 0.005 N H_2SO_4, applied at a flow rate of 0.8 mL/min.

3. Results and Discussion

3.1. Sequencing and analysis of lactate dehydrogenase

The D-*lactate dehydrogenase* (*ldh*A) gene of *E. aerogenes* ATCC 29007 was amplified using degenerate primers *ldh*F and *ldh*R. The sequence result obtained was analyzed using the BLAST program. The gene was found to be 99% identical to D-*lactate dehydrogenase* (*ldh*A) of *E. aerogenese* KCTC 2190 and 96% identical to that of *K. pneumonia* (ldhA). D-Lactate dehydrogenase (LDH) catalyzes the interconversion of pyruvate and lactate, and is a member of the 2-hydroxyacid dehydrogenase family. The NAD+ binding domain is inserted within the linear sequence of the mostly N-terminal catalytic domain, which has a similar domain structure to the internal NAD binding domain. Structurally, these domains are connected by extended alpha helices and create a cleft in which NAD is bound, primarily to the C-terminal portion of the 2nd (internal) domain.

3.2. Metabolic engineering of aerogenes ATCC 29007 to ferment bioethanol

Eliminating the pathways of undesired metabolites, lactate, acetate, 2,3-butanediol and succinate, is an ideal strategy to improve bioethanol productivity from glycerol. Here, we introduced the deletion of the D-*lactate dehydrogenase* (*ldh*A) gene in wild-type *E. aerogenes* ATCC 29007 since lactate is the major byproduct during bioethanol fermentation [31]. To delete the *ldh*A gene from the chromosome of *E. aerogenes* ATCC 29007, the pKM208 helper plasmid was transformed, and carbenicillin-resistant colonies were selected on LB-agar plates. The *ldhA* gene in *E. aerogenes* was targeted for knockout using the λ-Red recombination system. Then, kanamycin resistance cassette (1.7 kb) was transformed, and mutant strain *E. aerogenes* SUMI014 was screened on LB-agar plates containing 50 μg/mL kanamycin. PCR amplification of the chromosomal DNA from the respective mutant using primers *ldh*AF and *ldh*AR confirmed the correct genetic knockouts (Fig. 2). The deletion of *ldh*A gene gave 1.9 kb band, since deletion primers were designed 75 bps downstream of the start codon and 55 bps upstream of the stop codon. After deletion of *ldh*A gene, the kanamycin cassette was removed from chromosome by FRT recombination through transforming 706-FLP plasmid. The mutant *E. Aerogenes* SUMI014 was successfully obtained and used to examine metabolic variation in the following experiment.

Fermentation was carried out for both wild type *E. aerogenes* ATCC 29007 and engineered *E. aerogenes* SUMI014 in production media containing 60 g/L glycerol under fermentation conditions of pH 7 for 48 h at 37 °C. The fermentation of engineered *E. aerogenes*

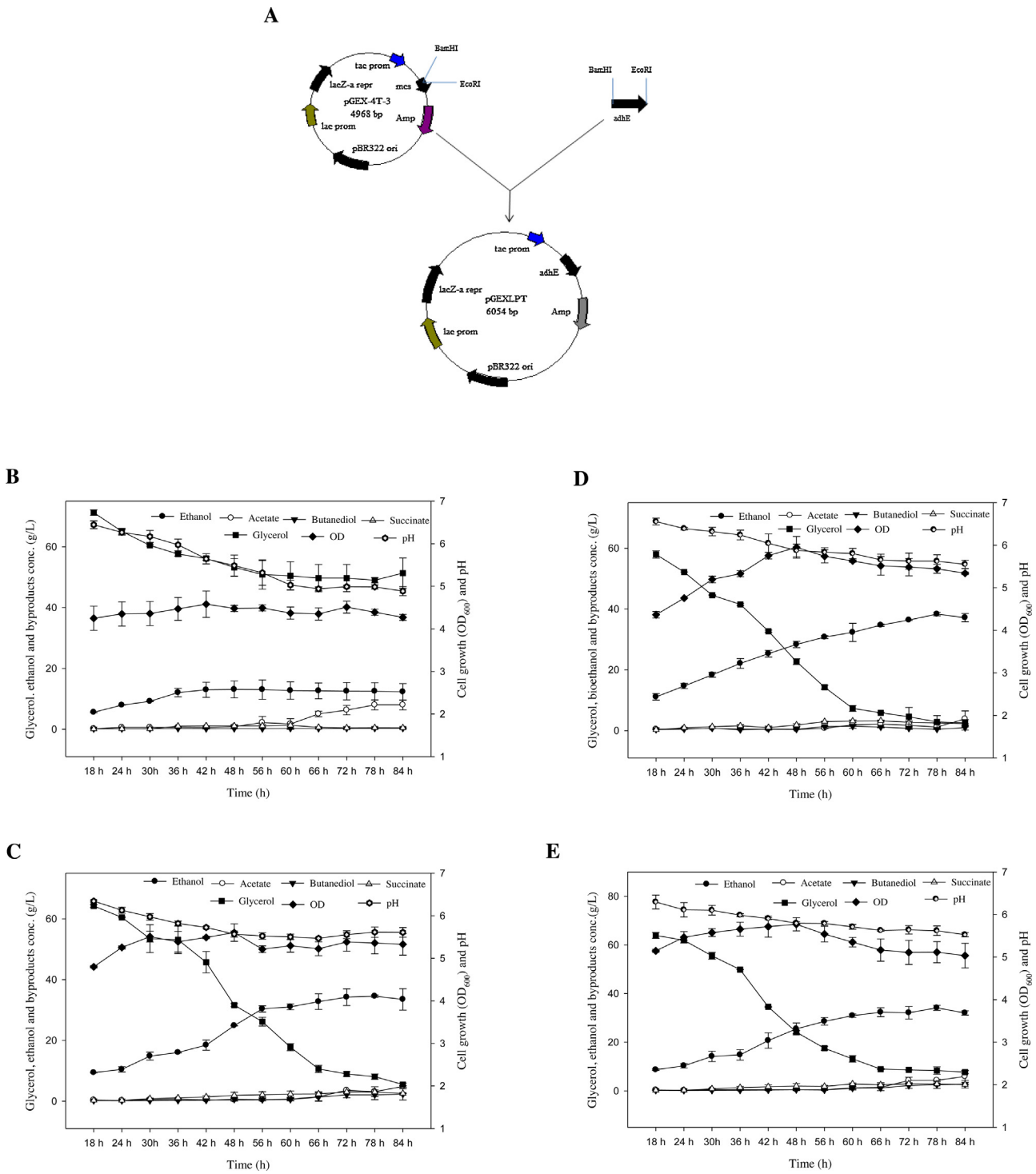

Fig. 6. Construction of recombinant plasmid pGEXLPT (A) and production of bioethanol by wild *E. aerogenes* ATCC 29007 (B), mutant *E. aerogenes* SUMI014 (C), recombinant mutant *E. aerogenes* SUMI2008 (D) and control strain with plasmid pGEX-4T-3 (E).

SUMI014 in aerobic conditions produced 21.42 g/L bioethanol, which is 7.9 g/L greater than that of 13.52 g/L by wild type *E. aerogenes* ATCC 29007 (Fig. 3A). Also, bioethanol production was almost one fold greater (6.70 g/L) by the mutant strain as compared with 3.84 g/L by the wild type counterpart in anaerobic conditions (Fig. 3C). Fig. 3C also shows that the glycerol was converted into bioethanol before 18 h of cultivation during anaerobic fermentation. Similarly, the production of succinic acid and 2,3-BDO was shown to be greater in the *ldh*A deleted mutant than in the wild-type (Fig. 3B and D). However, the production of lactic acid was decreased as compared with the wild type *E. aerogenes* ATCC 29007, confirming that the major lactate dehydrogenase (*ldh*A) gene was successfully deleted from the chromosome of *E. aerogenes* ATCC 29007. A decrease in acetic acid production was also observed in mutant strain when compared with the wild type counterpart. An interesting benefit of this *ldh*A deletion was an increase in microbial cell growth [22]. Generally, a metabolically engineered microorganism has reduced cell growth compared with the wild type

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strain [32,33]. The increased microbial cell growth of the mutant could be largely attributed to the reduced production of lactic acid, which lowers the acidification rate of the media [34]. Eventually, increase in cell growth of *E. aerogenes* SUMI014 favors an increase in bioethanol production up to certain concentration, however, when the ethanol concentration become maximum in culture broth the cell growth gets decreased. Deletion of *ldh*A gene increased the concentration of NADH and a redox balance be achieved. The greater availability of NADH led to a shift towards production of bioethanol, the major fermentation product [35]. Converti and Perego [36] also explained that amongst the intracellular metabolic pathways in *E. aerogenes*, lactate production was the most NADH-consuming pathway. Thus, the deletion of *ldh*A gene alters the metabolic flux and increases the production of the reduced metabolite, bioethanol.

3.3. Effect of temperature, time profile and initial medium pH

Physical parameters such as temperature and time profile are sensitive primary factors for the fermentation of microorganisms. Therefore, *E. aerogenes* SUMI014 was incubated in 100 mL production medium at 32 °C, 34 °C, and 37 °C for various time intervals by adjusting the medium to pH 7 at 200 rpm. *E. aerogenes* SUMI014 was grown very well at 34 °C and produced 22.96 g/L of bioethanol as compared with 21.73 g/L at 32 °C and 21.77 g/L at 37 °C after 48 h of cultivation (Fig. 4A).

Due to its influence on bacterial metabolism, pH level is one of the most important factors affecting cell growth and production of target products in bio-industry [37]. Due to the activity of living cells, various organic acids are produced and accumulated in culture medium. The resulting decrease in pH causes a significant decrease in bioethanol production. Here, the effect of the initial pH of the medium was investigated in order to determine the most suitable pH for the growth of *E. aerogenes* SUMI014 and bioethanol production. *E. aerogenes* SUMI014 was fermented at 34 °C for a period of 48 h in aerobic conditions in media adjusted to varying pH (value range 4–8.5) by the addition of hydrochloric acid and NaOH, accordingly. Fig. 4B shows that the concentration of bioethanol gradually increased with increasing pH and reached a maximum (28.65 g/L) at pH 7.5 for 48 h incubation. Production declined at higher pH values due to low substrate utilization and a high pH gradient between the intracellular and extracellular compartments. As the pH gradient increases, the proton-motive force decreases, and hence, more ATP is required for the extrusion of the same number of protons. Therefore, less ATP remains for biosynthesis and growth under aerobic conditions [38]. This means that glycerol utilization was limited by the pH gradient between the inside and the outside of the cells. The pH gradient prevented cells from transporting protons (H^+) and utilizing substrate. There was an inhibitory effect of pH on glycerol utilization and ethanol production by *E. aerogenes* in the pH range 6–7.5. In this pH range, *E. aerogenes* SUMI014 produced 17.70 ~ 24.50 g/L bioethanol by utilizing 80 ~ 95% glycerol of 80 g/L. This result can be explained by the ratio of reduced to oxidized nicotinamide adenine dinucleotide (the NADH/NAD^+ ratio), which affects the distribution of carbon flux through the metabolite routes under aerobic conditions. Nakashimada et al. [39], demonstrated the relationship between culture pH and NADH/NAD^+ ratios in various pH ranges. In the pH range 6–6.7, NADH/NAD^+ ratios were higher than at other pH values and this affected hydrogen production by *E. aerogenes*. From a reaction engineering perspective, a higher NADH/NAD^+ ratio benefits reactions in which NADH is used as a cofactor. *Enterobacter aerogenes* is known to utilize NADH as a cofactor for the production of ethanol and 2,3-butanediol. Zhang et al. [40] reported that ethanol production was enhanced by the addition of NADH. The findings that a change in growth medium pH affected growth and bioethanol production indicate that *E. aerogenes* SUMI014 is sensitive to changes in pH.

3.4. Effect of phosphate salts

Excessive changes in the hydrogen ion concentration (pH) of the medium were prevented by the addition of a phosphate salt (which acts a buffer). Therefore, the engineered strain, *E. aerogenes* SUMI014, was fermented with various concentrations of both KH_2PO_4 and K_2HPO_4 at a ratio (1:1) in order to determine the optimal concentration of phosphate for the production of bioethanol. Bioethanol production increased with increasing concentrations of both phosphate salts and reached a maximum (28.99 g/L) at a concentration of 6.5 g/L at 34 °C for 48 h and pH 7.5. Further increases in phosphate concentrations inhibited bioethanol productivity (Fig. 5). The effect of phosphate salts on ethanol production was investigated because the addition of phosphate salts improves the efficiency of the fermentation process by increasing the formation of adenosine triphosphate (ATP), which is the main source of energy in cells [41]. Starvation of *E. aerogenes* SUMI014 with respect to potassium, phosphate, or magnesium ions leads to a reversible increase in the rate of protein degradation and inhibition of ribonucleic acid (RNA) synthesis [42]. Therefore, the addition of phosphate salts in the production medium is required to increase the generation of ATP molecules by preventing protein degradation as well as the inhibition of RNA synthesis during bioethanol production.

3.5. Overexpression of adh*E* gene

The *adh*E gene of *E. aerogenes* KCTC 2190, which is 86 % identical to the alcohol dehydrogenase (*AdhY*) from *Citrobacter youngae* ATCC 29220 and 85 % identical to that of *E. coli* str. K-12 substr. MG1655 at the amino acid level, was cloned into plasmid pGEX-4T-3 under the control of the Tac promoter. ADH, a key enzyme required for the conversion of acetyl-CoA to ethanol in the presence of two molecules of NADH, was overexpressed in *E. aerogenes* SUMI014. Expression of the gene can be induced by adding 0.1 mM IPTG solution after 2 h of incubation. Then the proper expression of *adh*E gene was checked by measuring the ADH activity after 42 h of incubation in production media (Table 2). The enzyme data showed that the *adh*E overexpression recombinant mutant strain *E. aerogenes* SUMI2008 has highest enzyme activity as compared with other strains. On the other hand, mutant strain has higher ADH activity as compared with that of wild type strain because the deletion of *ldh*A gene increased the ADH activity [34]. The *adh*E-overexpressing recombinant mutant *E. aerogenes* SUMI2008, mutant *E. aerogenes* SUMI014, wild *E. aerogenes* KCTC 29007 and control strain containing only plasmid pGEX-4T-3 were incubated in 100 mL production media containing 80 g/L glycerol as a carbon source at 34 °C for 78 h. The *adh*E-overexpressed strain produced 38.32 g/L bioethanol, greater by an average of about 3.78 g/L than that of parental mutant strain of 34.54 g/L, and by 25.23 g/L than

Table 3
The titer, yield and productivity of bioethanol produced by *E. aerogenes* ATCC 29007, *E. aerogenes* SUMI014, *E. aerogenes* SUMI2008, and the control *E. aerogenes* SUMI014 strain with empty plasmid at optimized conditions.

Strains	Titer (g/L)	Yield (g/g)	Productivity (g/L h)
E. aerogenes ATCC 29007	13.09	0.1561	0.1614
E. aerogenes SUMI014	34.22	0.4278	0.4387
E. aerogenes SUMI2008	38.32	0.4792	0.4912
E. aerogenes SUMI014 with empty plasmid	33.34	0.4168	0.4274

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Table 4
Ethanol produced by different strains utilizing glycerol as a carbon source.

Strains	Substrates	Methods	Ethanol production		References
			Concentration (g/L)	Productivity [g/(L h)]	
E. coli EH05	Glycerol	Batch	20.7	0.22	Durnin et al. [43]
Enterobacter aerogenes ATCC 13048	Glycerol	Batch	20	0.16	Nwachukwu et al. [23]
Enterobacter aerogenes Hu-101	Glycerol	Batch	10	0.81	Ito et al. [44]
Klebsiella pneumonia M5a1	Glycerol	Fed-batch	18	0.28	Cheng et al. [45]
Klebsiella *pneumonia* GEM167/pBR-pdc-adh	Glycerol	Fed-batch	25	0.78	Oh et al. [46]
Saccharomyces cerevisiae mutant	Glycerol	Batch	2.4	0.05	Yu et al. [47]
Hansenula polymorpha mutant	Glycerol	Batch	2.74	0.34	Hong et al. [48]
Enterobacter aerogenes ATCC 29007	Glycerol	Batch	13.09	0.16	This work
Enterobacter aerogenes SUMI 014	Glycerol	Batch	34.54	0.43	This work
Enterobacter aerogenes SUMI2008	Glycerol	Batch	38.32	0.48	This work

that of the wild type of 13.09 g/L at 34 °C for 78 h (Fig. 6B–D). But, the transformation of only empty plasmid pGEX-4T-3 did not affect the production of bioethanol as compared with that of the parental mutant strain (Fig. 6E). The titer, yield, and productivity of all constructed strains at optimized conditions were calculated (Table 3). The bioethanol yield in case of *E. aerogenes* SUMI2008 was highest 0.48 g/g as compared with 0.43 g/L by the mutant, 0.42 g/g by the control and 0.16 g/g by the wild type. Similarly, the bioethanol productivity was also highest in case of *E. aerogenes* SUMI2008 (0.49 g/L h) as compared with 0.44 g/L h of the mutant, 0.43 g/L h of the control and 0.16 g/L h of the wild strain. The production of bioethanol increased with time and reached a maximum after 78 h of fermentation, in case of recombinant *E. aerogenes* SUMI2008 and mutant *E. aerogenes* SUMI014, and eventually started to decrease. In the case of wild type *E. aerogenes* ATCC 29007, however, bioethanol production reached maximum after 48 h of fermentation and subsequently decreased. The increase in the fermentation life time of the mutant strain may be due to reduced production of lactic acid, subsequently increasing the availability of NADH molecules. The availability of NADH molecules in cells favors the conversion of more molecules of acetaldehyde into bioethanol by *adh*E gene [36]. NADH, generated by the oxidation of dihydroxyacetone phosphate to phosphoenolpyruvate, is then converted to NAD^+ during the reduction of acetaldehyde to bioethanol. NADH is produced by the pyruvate kinase reaction. It is important for organisms that undergo fermentation to maintain a redox balance as alcohol dehydrogenase oxidizes NADH to NAD^+ so that the glycolytic pathway may continue. We also compared bioethanol production from glycerol by different ethanolic strains (Table 4). The highest bioethanol producing strain was found to be *E. aerogenes* SUMI2008, amounting to 38. 32 g/L at 34 °C after incubating for 78 h, as compared with 20 g/L bioethanol, produced by mutant *E. aerogenes* ATCC 13048 at 37 °C after incubating 48 h [23]. This suggests that *E. aerogenes* SUMI2008 is the best strain for the production of bioethanol through genetic manipulation.

4. Conclusions

This experiment demonstrated the metabolic engineering of *E. aerogenes* ATCC 29007 to enhance the production of bioethanol and valuable chemicals. The mutant strain *E. aerogenes* SUMI014, generated by deleting the D-*lactate dehydrogenase* (*ldh*A) gene, enhanced the production of bioethanol by 1.5 fold as compared with that of the wild *E. aerogenes* ATCC 29007. This experiment also demonstrated that the deletion of the D-*lactate dehydrogenase* (*ldh*A) reduced the production of lactate by 2.5 fold and increased the fermentation life time. This may be due to the fact that the deletion of the D-*lactate dehydrogenase* (*ldh*A) gene increases the concentrations of carbon flux and NADH, favoring the production of reduced metabolites such as bioethanol, succinate and 2,3-butanediol. However, the conversion rate of glycerol into bioethanol was very low in anaerobic conditions compared with the aerobic conditions, which may be due to the slow growth rate of bacterial cells in anaerobic conditions. By using a combination of *ldh*A gene deletion and *adh*E gene overexpression, bioethanol production was maximized. The results obtained in this experiment will provide valuable guidelines for further engineering of bioethanol producers.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LPT performed overall experiments containing experimental design, metabolic engineering and wrote the manuscript. SJL, JHL YX and HSC helped for the preparation of media and fermentation experiments. CP and SWK coordinated the experimental design and reviewed the manuscript. All authors read and approved the final manuscript.

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