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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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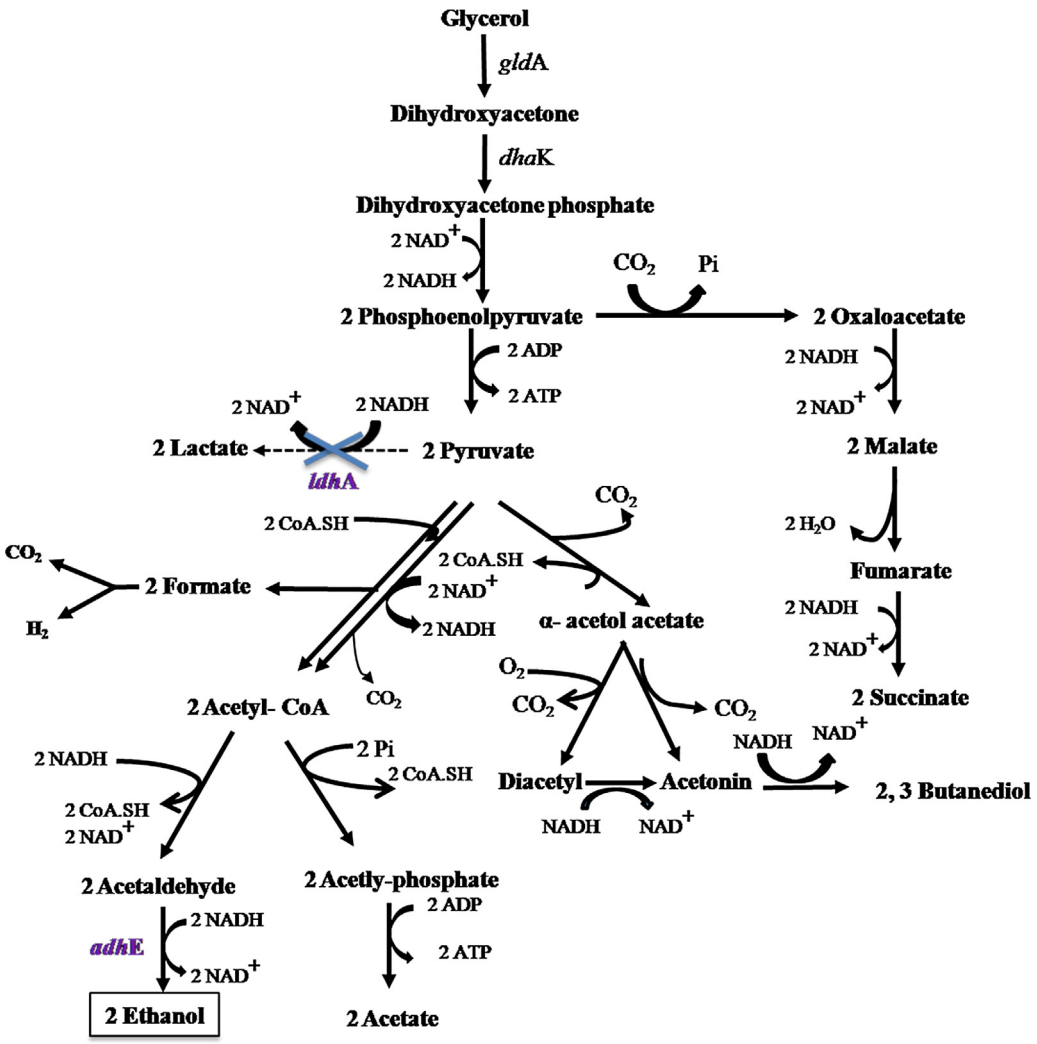

Fig. 1. Fermentative pathway of glycerol in *E. aerogenes* ATCC 29007 for the production of bioethanol and byproducts in aerobic conditions. Where, *ldh*A is, D-*lactate dehydrogenase*, and *adh*E is, *alcohol dehydrogenase* genes.

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.