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ORIGINAL ARTICLE

A single metabolite production by *Escherichia coli* BW25113 and its *pflA.cra* mutant cultivated under microaerobic conditions using glycerol or glucose as a carbon source

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Abstract Abundant, low prices and a highly reduced nature make glycerol to be an ideal feedstock for the production of reduced biochemicals and biofuels. *Escherichia coli* has been paid much attention as the platform of microbial cell factories due to its high growth rate (giving higher metabolite production rate) and the capability of utilizing a wide range of carbon sources. However, one of the drawbacks of using *E. coli* as a platform is its mixed metabolite formation under anaerobic conditions. In the present study, it was shown that ethanol could be exclusively produced from glycerol by the wild type *E. coli*, while D-lactic acid could be exclusively produced from glucose by *pflA.cra* mutant, where the glucose uptake rate could be increased by this mutant as compared to the wild type strain. It was also shown that the growth rate is significantly reduced in *pflA.cra* mutant for the case of using glycerol as a carbon source due to redox imbalance. The metabolic regulation mechanisms behind the fermentation characteristic were clarified to some extent.

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1. Introduction

Present global energy requirements are fulfilled primarily via fossil fuel combustion, and thus the world is dependent on a non-renewable resource for its energy needs [10]. The increasing economic growth and prosperity have been accelerated

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worldwide with an increasing demand on energy mostly generated from fossil fuels. This has brought the rapid global warming caused by the emission of green-house gases such as CO_2, resulting in a disastrous climate change, where this problem is becoming crucial. Currently the importance of an alternative energy source has become even more necessary, not only due to the expected depletion of the limited fossil fuel stock, but also for a much safer and better environment, and there has also been an increasing interest worldwide in seeking alternative sources of energy [7,11,16,17,32,34].

It is, therefore, important to consider the alternative renewable sources for energy and chemical production. In particular, biomass-oriented fuels and chemicals seem to be most promising. Although extensive investigations have, therefore, been made on biofuel and biochemical production from biomass resources, the main problem is the economic feasibility [36]. It is, therefore, highly desirable to utilize the cheap raw materials for fermentative production of such products.

Since glycerol is the byproduct of biodiesel production [26,36] it is preferred as a low cost and abundant substrate for the production of bio-chemicals and biofuels [3]. Glycerol has a highly reduced nature when compared to other sugars such as glucose, xylose, etc., which indicates that glycerol may be more useful for the production of succinate, ethanol, lactate, and diols [36].

Among biofuels, bioethanol has been extensively investigated, even in an industrial scale in Brazil and USA, where ethanol can be produced by fermentation of food stocks, such as corn, sugarcanes and sugar beets. In 1925, Henry Ford had quoted ethyl alcohol, ethanol, as 'the fuel of the future' [2]. Although *Saccharomyces cerevisiae* has been extensively used for ethanol fermentation due to ethanol tolerance [14,18] and it can utilize hexose sugars such as glucose, it does not have the capability of utilizing pentose sugars and others; therefore, the major drawback is the narrow range of its capability of assimilating carbon sources.

On the other hand, most bacteria such as *Escherichia coli* can assimilate a broad range of carbon sources including hexoses, pentoses, and others. *E. coli* is a gram-negative, facultative anaerobic and non-sporulating bacterium [33] and it is the most widely used prokaryotic system which produced heterologous proteins for the industrial production of bacterial metabolites by batch and fed-batch operations [15,37]. *E. coli* has been regarded as the workhorse of modern biotechnology [12] for the potential microbial production of biofuels and biochemicals.

Biofuel and biochemical production by recombinant *E. coli* has been paid much attention due to its high growth rate (contributing to the productivity) and a broad range of carbohydrate utilization [22]. In the present study, therefore, the fermentative utilization of glycerol by *E. coli* is considered as compared to glucose.

One of the drawbacks of using *E. coli*, is the mixed metabolite production (such as formate, lactate, acetate, succinate, and ethanol) under micro-aerobic or anaerobic conditions, where it lowers the yield of the target metabolite and gives burden for the downstream processing. It is highly desirable to produce a single metabolite in practice. In fact, this may be attained by the specific pathway mutation. For example, *pfl* gene knockout mutation allows the exclusive D-lactate production from glucose in *E. coli* to be cultivated under anaerobic condition [38].

Another important factor for the useful metabolite production is the substrate uptake rate. In particular, it is highly desirable to increase the glycolytic flux to yield higher pyruvate formation, where pyruvate is the starting metabolite for a variety of target metabolite formations. The carbon flow in *E. coli* is controlled by Global regulator Cra (catabolite repressor/activator) [21,29]. Its control mechanism is cAMP-independent [5], where Cra represses the expressions of the sugar uptake genes such as *ptsHI*, and the glycolysis genes such as *pfkA*, *pykF*, *zwf*, *edd*, *eda* as well as TCA cycle gene *acnB*, while it activates the gluconeogenic genes such as *fbp*, *ppsA*, *pckA*, the glyoxylate pathway gene *aceA* and the TCA cycle genes such as *icdA* and *acnA* [28]. The set of such genes implies that Cra activates the gluconeogenic pathway genes and represses the glycolysis genes. This implies that the glycolytic flux may be increased by *cra* gene knockout [30,35], where acetate is overproduced in *cra* mutant, since the expression of *aceA* and *icdA* is repressed [30]. This problem may be avoided in *pfl* mutant, cultivated under anaerobic conditions, since AcCoA formation is blocked by this mutation.

In the present investigation, we considered the single metabolite production such as ethanol (biofuel) production by the wild type *E. coli*, and D-lactic acid (biochemical) production by *pflA.cra* mutant using either glucose or glycerol as a carbon source.

2. Materials and methods

2.1. Strains used

The strains used in the present study were *E. coli* BW25113 ($lacI^q rrnB_{T14}$ $\Delta lacZ_{wJ16}$ *hsdR514* $\Delta araBAD_{AH33} \Delta rha BAD_{LD78}$), and its *pflA.cra* double gene knockout mutant, where the mutant was constructed based on the method of Datsenko and Wanner [4].

The gene knockout mutant was constructed at Keio University, and open to the public as KEIO collection [1]. The double-gene knockout mutant was constructed in the similar method. The basic knockout strategy is to replace a *cra* gene of the *pflA* gene knockout mutant (kanamycin resistant) with a selectable antibiotic (ampicillin) resistant gene (*amp*) that is generated by PCR using primers with homology extensions. After selection, the resistant gene can be eliminated using a helper plasmid. The mutant was verified by comparing the length of the PCR amplified fragments with the expected length from the genome database.

2.2. Media compositions

Micro-aerobic batch culture was carried out using M9 synthetic medium containing the following components: 48 mM Na_2HPO_4, 22 mM KH_2PO_4, 10 mM NaCl and 30 mM $(NH_4)_2SO_4$. The carbon source was either glucose (10 g/L) or glycerol (20 g/L) for the micro-aerobic batch culture. The following components were filter sterilized and then added (per liter) with 1 ml of 1 M $MgSO_4$, 1 ml of 0.1 mM $CaCl_2$, 1 ml of 1.0 mg/L Vitamin B_1 and 10 ml of trace element solution containing (per liter): 0.55 g $CaCl_2{\cdot}2H_2O$, 1.67 g $FeCl_3{\cdot}6H_2O$, 0.10 g $MnCl_2{\cdot}4H_2O$, 0.17 g $ZnCl_2$, 0.043 g $CuCl_2{\cdot}2H_2O$, 0.06 g $CoCl_2{\cdot}2H_2O$ and 0.06 g $Na_2MoO_4{\cdot}2H_2O$.

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