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Conversion of Glucose and Gluconate to Ethanol in Mineral Salts Medium using Recombinant *Escherichia coli* Strains

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Abstract

Escherichia coli AH003, a derivative of *E. coli* KO11 with the L-lactate dehydrogenase (ldh) and pyruvate formate lyase (*pfl*) genes deleted and its parent strain *E. coli* KO11 were used as the ethanologen to convert glucose and gluconate to ethanol in M9 minimal medium. *E. coli* AH003 grew very poorly on glucose in M9 medium. However it achieved rapid growth when gluconate was used as the carbon source. The addition of gluconate to medium containing glucose improved the rate of glucose utilization. In contrast, *E. coli* KO11 grew well on both glucose and gluconate in M9 medium. The addition of gluconate to medium containing glucose did not improve the rate of glucose utilization. We believe that the deletion of the pfl gene in *E. coli* AH003 led to the different fermentation results. The co-fermentation of gluconate and glucose could be a useful strategy to improve the rate of glucose fermentation and decrease nutrient requirements for engineered strains lacking the pfl gene and grown under anaerobic conditions.

Keywords: Gluconate; Ethanol; *Escherichia coli*; Fermentation; Medium

Introduction

Cellulosic biomass is an attractive, renewable, abundant, and low-cost feedstock for fuel and chemical production [1]. Their biochemical conversion features cellulase mediated hydrolysis and microbial fermentation. The cellulosic biomass hydrolysate mainly contains hexose and pentose sugars, which can be fermented to various fermentation products including ethanol. Recently, copper-dependent lytic polysaccharide monooxygenases (LPMOs) were found to be able to catalyze the oxidative cleavage of cellulose, generating oxidized cellodextrins in concert with cellobiose dehydrogenases [2]. The addition of LPMOs to cellulase enzyme cocktails to enhance cellulose degradation generates cellobionic acid and gluconic acid in addition to pentose and hexose sugars in the hydrolysate [3]. Our lab proposed an alternative route for biofuel and chemical production from cellulosic biomass in which cellobionate instead of monomeric sugars is produced as the reactive intermediate for subsequent fermentation [4]. Conversion of gluconic acid or cellobionic acid to biofuels is of importance for complete utilization of all the carbon sources available in biomass.

Both of the hydrolysis products from cellobionate, glucose and gluconate, were found to be utilized efficiently by *E. coli* KO11 [4]. From 1 mole of gluconate, 1.5 moles of ethanol and 0.5 moles of acetate were produced, while 2 moles of ethanol were produced from 1 mole of glucose. The rate of gluconate utilization by *E. coli* KO11 was even faster than that of glucose in Luria Broth (LB) medium [4]. Glucose and gluconate were utilized by *E. coli* KO11 simultaneously when both of them were present as the carbon source, and again gluconate was used faster than glucose. Although *E. coli* KO11 was an excellent ethanologen for ethanol production from glucose, and close to theoretical yield was achieved from glucose in LB medium, the ethanol yield from gluconate produced by this strain was about only 87% of the theoretical maximum [4,5]. *E. coli* KO11 was then engineered for improved ethanol production from gluconate. Deletion of the gene encoding pyruvate formate lyase (pfl) alone led to lower acetate yield, lower ethanol yield and more carbon flow to lactic acid production. Subsequent deletion of the L-lactate dehydrogenase (ldh) gene in addition to pfl eliminated lactate production, reduced the carbon flow toward acetate production, and improved the ethanol yield from 87.5% to 97.5% of the theoretical maximum. The resulting strain was named *E. coli* AH003 [6].

All previous work with *E. coli* AH003 has used complex growth media containing laboratory nutrients such as yeast extract and tryptone. The use of these nutrients for the industrial production of ethanol may not be feasible because of their high cost. In this study, we focus on the fermentation of glucose and gluconate to ethanol using a minimal mineral salts medium. Parent strain *E. coli* KO11 was used as a comparison.

Materials and Methods

Bacterial strains and media

Strains *E. coli* KO11 and *E. coli* AH003 were stocked at -80˚C. Before each fermentation study, each strain was streaked onto a LB plate and incubated at 37˚C overnight. Cells from a single colony were inoculated into a 15 mL centrifuge tube containing 8 mL LB. The culture was incubated at 37˚C for 6 hours. 1 mL of the pre-culture was transferred to a 200 mL seed serum bottle containing 100 mL of LB

with 2% glucose. The seed culture was incubated for 14 hours at 37 °C and shaken at 200 rpm. Cells were harvested by centrifugation and used to inoculate fermentation serum bottles, which contained 100 mL of medium with various carbon sources, to a starting OD600 of 0.05.

Two media were used in this study. They are LB medium and M9 medium. LB medium contained the following components: 20 g/L tryptone, 10 g/L yeast extract, and 10 g/L sodium chloride. M9 medium contained 6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 1 g/L NH_4Cl, 0.5 g/L NaCl, 0.002 M $MgSO_4.7H_2O$, 0.0001 M $CaCl_2$, and 1 mg/L thiamine-HCl. Glucose and potassium gluconate were added at various starting concentrations as the carbon source.

Anaerobic fermentation and growth conditions

The serum bottles were pH adjusted to 6.5 with hydrochloric acid. Bottles were purged with argon to obtain anaerobic conditions. All fermentations were run in a minimum of triplicates on a rotary shaker at 37 °C and 200 rpm using 200 mL serum bottles containing 100 mL of fermentation broth. Samples were taken out at various time intervals. The cell density was measured at 600 nm using a spectrophotometer. The concentrations of sugars, sugar aldonates, and fermentation products were analyzed using HPLC.

Analytical procedures

The concentrations of glucose, gluconate, acetic acid, and ethanol were measured by a Shimadzu HPLC equipped with a refraction index detector, PDA detector, and a Transgenomic ICSep ION-300 column (Transgenomic, San Jose, CA, USA) at 80˚C. Five millimolar sulfuric acid at a flow rate of 0.6 mL/min was used as mobile phase. To measure the glucose and gluconate concentration in the glucose and gluconate co-fermentation experiments, the supernatant of the fermentation broth was analyzed using a Transgenomic CARBOSep COREGEL-87C (Transgenomic, San Jose, CA, USA) column at 80˚C. 20 mM calcium chloride at a flow rate of 0.6 ml/min was used as the mobile phase.

Results

Co-fermentation of glucose and gluconate in LB medium

Figure 1 shows the fermentation of 50 mM of glucose, gluconate and the mixture of gluconate and glucose to ethanol and acetate in the LB medium. AH003 completely utilized all 50 mM gluconate in 5.5 hours, while it took 8.5 hours to completely consume 50 mM of glucose. When both 50 mM of gluconate and 50 mM of glucose were co-fermented, it took about the same 5.5 hours to deplete all the gluconate and 8.5 hours to deplete all the glucose. The rates of glucose and gluconate utilization in the mixture were almost the same as when the other substrate was not present. The yield of ethanol from glucose was about 100%, the yield of ethanol from gluconate was about 97%, the ethanol yield from glucose and gluconate mixture was about 97%.

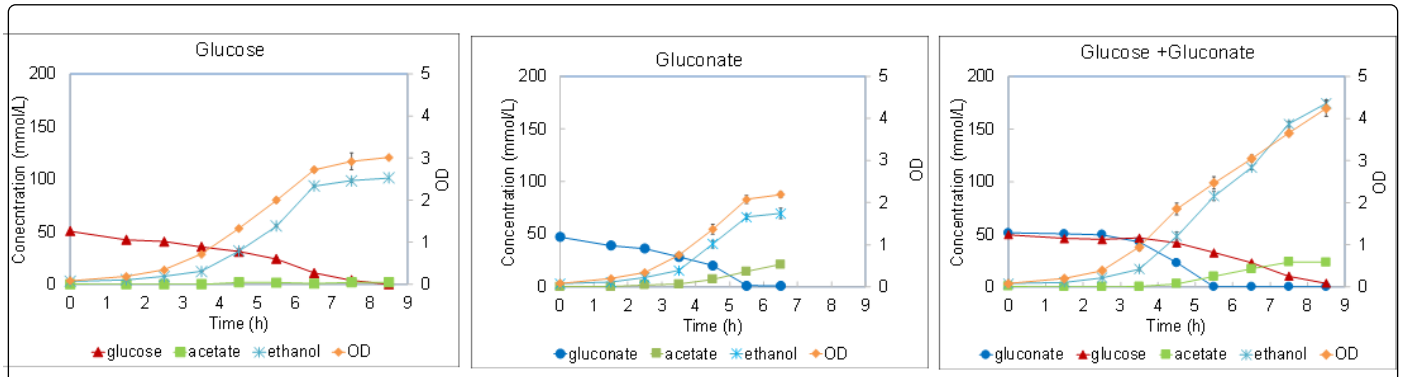

Figure 1: Time course of substrate utilization and product formation by *E. coli* AH003 strain in LB medium a) glucose, b) gluconate, c) glucose and gluconate. Error bars indicate standard deviations of the three repeats.

Fermentation of glucose, gluconate and the glucose and gluconate mixture in the mineral salts medium

The conversion of glucose, potassium gluconate and the mixture of glucose and potassium gluconate were evaluated using M9 mineral medium without any casamino acid addition. As shown in Figure 2, M9 mineral salts medium supported the growth of AH003 on gluconate. All gluconate was completely consumed within 17 hours, and the OD of the strain reached 1.0. About 62 mM of ethanol and 16 mM of acetate were produced from 50 mM of the gluconate. Ethanol yield was about 82% of the theoretical maximum. When glucose was used as the carbon source, the strain grew very slowly. The optical density of the strain was only about 0.2, and only one third of the glucose was consumed after 32 hours. The ethanol yield from the consumed glucose was about 80% of the theoretical maximum. Interestingly, the strain used glucose much faster in the 50 mM glucose and 50 mM gluconate mixture than when glucose was present alone. All glucose was used in 30 hours, while all gluconate was used in 27 hours. The optical density of the culture reached 1.6. About 139 mM ethanol was produced from the mixture, yielding 80% of the maximal theoretical yield. In the fermentation using AH003, no obvious succinate, lactate nor formate were detected in the fermentation broth, which aligned with the fact that the genes responsible for generating them such as frd, ldh and pfl were knocked out in the *E. coli* AH003 genome.

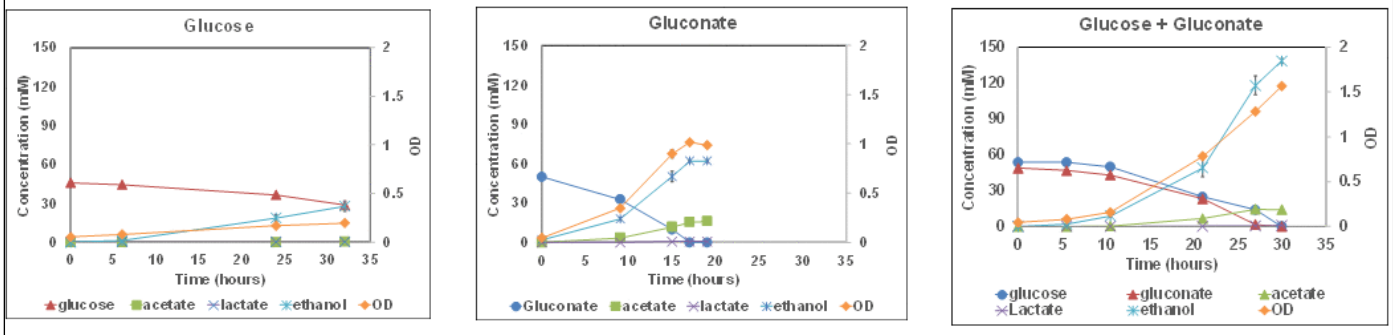

Figure 2: Time course of substrate utilization and product formation by *E. coli* AH003 strain in M9 medium a) glucose, b) gluconate; and c) glucose and gluconate. Error bars indicate standard deviations of the three repeats.

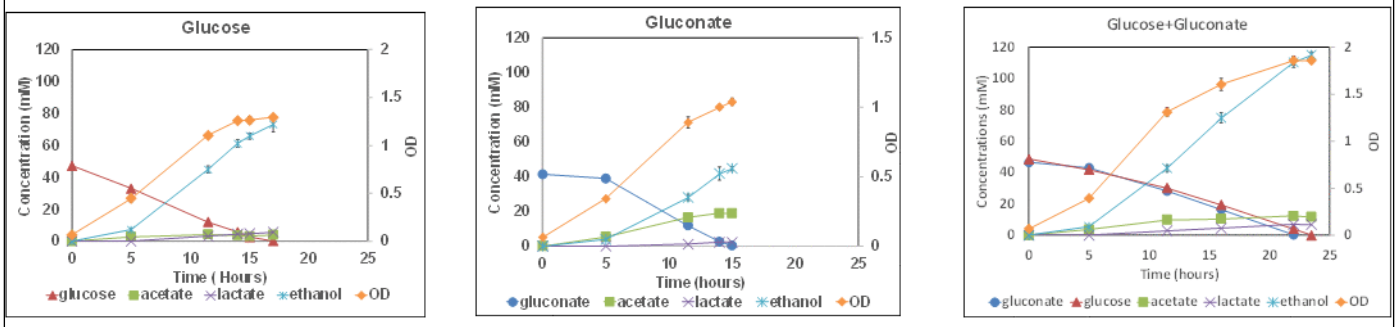

Figure 3: Time course of substrate utilization and product formation by *E. coli* KO11 strain in M9 medium a) glucose, b) gluconate; and c) glucose and gluconate. Error bars indicate standard deviations of the three repeats.

When *E. coli* KO11 was used as the ethanologen in M9 medium, we found that glucose was utilized not much slower than gluconate as shown in Figure 3. All glucose was used up in 16 hours, while all gluconate was used up in about 15 hours. Again, in the glucose and gluconate mixture, gluconate was used up in 22 hours, while glucose got used up in 23.5 hours. The addition of gluconate to glucose did not increase the rate of glucose utilization. The yields of ethanol from glucose, gluconate and their mixture were all about 75% of theoretical maximum. About 25-30 mM formate and about 5 mM lactate was produced in the fermentation broth. The rates of substrate utilization in all cases were faster than those of AH003.

Discussion

E. coli KO11 is one of the best ethanologens available to convert all biomass sugars such as glucose, xylose and arabinose to ethanol [7]. It contains chromosomally integrated genes encoding pyruvate decarboxylase (pdc) and alcohol dehydrogenase (adhB) from the Zymomonas mobilis PET operon [5]. The fumarate reductase (frd) gene was deleted to prevent succinate production and further improve the carbon flow toward ethanol production [5]. *E. coli* strains have distinct pathways to transport glucose and gluconate [8-11]. Gluconate does not seem to have any obvious effect on carbon catabolite repression of glucose and vice versa. The utilization of glucose and gluconate happens simultaneously. At low concentrations, the rates of glucose and gluconate utilization in co-fermentation were the same as when the other substrate was not present. When starting concentrations of glucose and gluconate in the co-fermentation were higher (100 mM), the substrate utilization rate seemed to be slowed by one another, perhaps due to higher concentrations of inhibitory metabolites produced from higher starting substrate concentrations [4].

The deletion of pfl and ldh in *E. coli* KO11 yielded the strain *E. coli* AH003 [6]. Although the deletion of pfl and ldh did not have any obvious effect on ethanol yield from glucose in LB medium [6], it significantly affected the strain's growth on glucose in minimal salts medium. *E. coli* AH003 can barely grow on glucose. However, both strain AH003 and KO11 maintained good growth when gluconate was used as the substrate. In contrast to the poor growth on glucose in M9 medium, *E. coli* AH003 utilized glucose much faster when both glucose and gluconate were present. When KO11 was used as the ethanologen, the presence of gluconate did not improve the rate of glucose utilization in the glucose and gluconate co-fermentation.

These results can be explained by the absence of the *pfl* gene in the AH003 genome. PFL and pyruvate dehydrogenase (PDH) are both responsible for acetyl-CoA production in *E. coli*. PDH is an enzyme that is strongly inhibited by NADH and requires NAD+ for the activity [12]. It has minimal functionality under anaerobic conditions when the ratio of NADH to NAD+ is high [12], as PFL is the main route for acetyl-CoA production under anaerobic conditions. However, recent studies have shown that there is still flux through the PDH pathway for certain strains of *E. coli* under anaerobic conditions, which serves to maintain redox balance and provide reducing equivalents when

glucose is the carbon source [13]. In *E. coli* AH003, the *pfl* gene was deleted. Thus, the PDH route is the only way for the cell to generate acetyl-CoA, which is the precursor for anabolism. When glucose, which is a much reduced substrate, was used, a high NADH/NAD+ ratio was generated which led to strong inhibition of the PDH and slower generation of acetyl-CoA. Consequently, AH003 grew on and fermented glucose very slowly. However, when gluconate was used as the substrate, a lower NADH/NAD+ level was achieved inside the cell since it is a more oxidized substrate [14]. As a result, PDH was less inhibited and normal growth and reasonable rate of gluconate utilization was achieved. When both glucose and gluconate were present in the co-fermentation, they were utilized simultaneously. The ratio of NADH/NAD+ should fall between the levels when glucose or gluconate were present alone. Hence, the gluconate utilization rate in the mixture was slower than that of gluconate alone, whereas the rate of glucose utilization was faster than that of glucose alone.

E. coli KO11 has an active PFL pathway. The formation of large amount of formate suggested that the *pfl* gene was active and the acetyl-CoA was generated through the PFL pathway instead of the PDH pathway. Since the ratio of NADH/NAD+ did not significantly affect PFL, nor acetyl-CoA production and anabolism, the fermentation of glucose occurred not much slower than gluconate. The effect of gluconate enhancing the rate of glucose utilization was not seen in the glucose and gluconate co-fermentation in *E. coli* KO11. However, the ethanol yields achieved using *E. coli* KO11 were lower than those of *E. coli* AH003 due to the formation of other metabolites.

Productivity and nutrient costs are both significant factors in process economics for the microbial production of commodity products. In this sense, gluconate is a very desirable and unique substrate. In all media tested, gluconate was utilized faster than glucose. Besides, gluconate needs less rich supplemental nutrients than glucose to support *E. coli* AH003's growth and product formation. The addition of gluconate could improve the glucose utilization rate in co-fermentation in minimal mineral salts medium. The deletion of the *pfl* gene to redirect carbon flow toward the target products is a very common strategy used in strain engineering for biofuels and chemicals production. We expect that what we observed with *E. coli* AH003 is likely applicable to strains with similar features too. Strains with *pfl* deleted normally need relatively rich media to support anaerobic growth and product formation [15,16]. The co-fermentation of gluconate and glucose could be a useful strategy to improve the rate of glucose fermentation and decrease nutrient requirements for engineered strains lacking the *pfl* gene grown under anaerobic conditions.

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