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BIOTECHNOLOGY

Homoethanol Production from Glycerol and Gluconate Using Recombinant *Klebsiella oxytoca* Strains

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ABSTRACT Gluconic acid, an oxidized cellulose degradation product, could be produced from cellulosic biomass. Glycerol is an inexpensive and renewable resource for fuels and chemicals production and is available as a byproduct of biodiesel production. Gluconate is a more oxidized substrate than glucose, whereas glycerol is a more reduced substrate than glucose. Although the production of homoethanol from glucose can be achieved, the conversion of gluconate to ethanol is accompanied by the production of oxidized byproduct such as acetate, and reduced byproducts such as 1,3-propanediol are produced, along with ethanol, when glycerol is used as the carbon source. When gluconate and glycerol are used as the sole carbon source by *Klebsiella oxytoca* BW21, the ethanol yield is about 62 to 64%. Coutilization of both gluconate and glycerol in batch fermentation increased the yield of ethanol to about 78.7% and decreased by-product accumulation (such as acetate and 1,3-propanediol) substantially. Decreasing by-product formation by deleting the *pta*, *frd*, *ldh*, *pflA*, and *pduC* genes in strain BW21 increased the ethanol yield to 89.3% in the batch fermentation of a glycerol-gluconate mixture. These deletions produced the strain *K. oxytoca* WT26. However, the utilization rate of glycerol was significantly slower than that of gluconate in batch fermentation. In addition, substantial amounts of glycerol remain unutilized after gluconate was depleted in batch fermentation. Continuous fed-batch fermentation was used to solve the utilization rate mismatch problem for gluconate and glycerol. An ethanol yield of 97.2% was achieved in continuous fed-batch fermentation of these two substrates, and glycerol was completely used at the end of the fermentation.

IMPORTANCE Gluconate is a biomass-derived degradation product, and glycerol can be obtained as a biodiesel byproduct. Compared to glucose, using them as the sole substrate is accompanied by the production of by-products. Our study shows that through pathway engineering and adoption of a fed-batch culture system, high-yield homoethanol production that usually can be achieved by using glucose as the substrate is achievable using gluconate and glycerol as cosubstrates. The same strategy is expected to be able to achieve homofermentative production of other products, such as lactate and 2,3-butanediol, which can be typically achieved using glucose as the substrate and inexpensive biodiesel-derived glycerol and biomass-derived gluconate as the cosubstrates.

KEYWORDS ethanol, fed-batch, gluconate, glycerol

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Cellulosic biomass is an attractive, low-cost, abundant, and renewable resource for fuels and chemicals production; cellulose, hemicellulose, and lignin are the three major constituents (1, 2). Biochemical conversion of lignocellulosic biomass to fuels and chemicals featuring enzymatic hydrolysis produces sugars for subsequent conversion (3). Cellulase cost remains one of the key bottlenecks in low-cost processing technology (4, 5). Recently, lytic polysaccharide monooxygenase (LPMO) was found to accelerate cellulose degradation (6, 7). The addition of LPMOs to the cellulase cocktail as an auxiliary enzyme can greatly increase both the efficiency of cellulose hydrolysis and the sugar yields (8). However, since LPMOs catalyze the oxidative hydrolysis of cellulose, oxidized cellulose degradation products, including cellobionic acid and gluconic acid, also accumulate in the cellulosic biomass hydrolysate by up to 4% (9, 10). If these degradation products are not converted to final products, they represent a loss of sugar in the hydrolysis process. In our previous study, we proposed a novel biochemical route for fuel and chemical production in which cellobionate would be directly produced from cellulose by an engineered fungal strain without any enzyme addition (11). Cellobionate and its hydrolysate (including both glucose and gluconate) could serve as the carbon sources for subsequent fuel and chemical production (12, 13).

Gluconate can serve as a carbon source for production of fuels and chemicals, including ethanol, isobutanol, pyruvate, triacylglycerol, and polyhydroxyalkanoates (14–18). Specifically, gluconate can be utilized by *Escherichia coli* and other bacteria via both the pentose phosphate (PP) pathway and the Entner-Doudoroff (ED) pathway (19, 20). As shown in Fig. 1A, when gluconate is metabolized to two pyruvates via the ED pathway, only one NADH is generated; subsequent conversion of the two pyruvates to two ethanols requires at least two NADHs. As a result, oxidized products such as acetate must be produced, along with ethanol, to maintain the overall redox balance. In the recombinant ethanologen *Klebsiella oxytoca* P2, which has the *Zymomonas mobilis* ethanol production pathway genes (PET operon), including pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase (*adhB*), inserted into the pyruvate formate lyase (*pflB*) site in the chromosome (21), the conversion of pyruvate to ethanol is predominantly via the pyruvate decarboxylase (PDC) pathway and consumes only one NADH. The maximum yield of ethanol from gluconate on per pyruvate basis is only 75% (13). When gluconate is metabolized via the PP pathway in *K. oxytoca* P2, 1 gluconate generates 5/3 pyruvates, 1 NADPH, and 5/3 NADHs. The subsequent conversion of the 5/3 pyruvates to ethanol could consume 5/3 NADHs generated during pyruvate production via the PDC pathway. If the 1 NADPH generated can be used for biosynthesis or other purposes, the theoretical maximal ethanol yield achieved via PP pathway could be close be 100% (20). When the rich medium such as Luria-Bertani (LB) broth is used, the reducing power demand for biosynthesis is rather low, and gluconate is expected to be mainly metabolized via the ED pathway (13).

When *E. coli* KO11—an engineered ethanologen—was used to produce ethanol from gluconate, the actual ethanol yield from gluconate was only 65.6% (14, 15). Lactate and acetate were the two major by-products. Hildebrand et al. deleted genes that encode the competing pathways, eliminating lactate production, reducing acetate production, and improving the ethanol yield to 73.1% (14). Acetate was produced as the main oxidized product, and its yield was about 17%. This metabolite profile supported that the gluconate was metabolized mainly via the ED pathway.

Utilization of second inexpensive substrates as cosubstrates is an attractive strategy to achieve metabolic pathway coordination and to increase product yield. We propose to utilize glycerol as a cosubstrate to gluconate to increase the ethanol yield. Glycerol, a biodiesel production by-product, is a renewable and inexpensive resource for fuels and chemicals production (22). Because of the low cost of glycerol as a feedstock, the cost of ethanol production from glycerol could be reduced by 40% compared to ethanol production from corn-derived sugars (23).

E. coli does not fermentatively utilize glycerol. However, *K. oxytoca* can. Glycerol is metabolized in *K. oxytoca* via an oxidative pathway and a reductive pathway (24). In the oxidative pathway, glycerol is first converted to pyruvate, generating two NADH; then

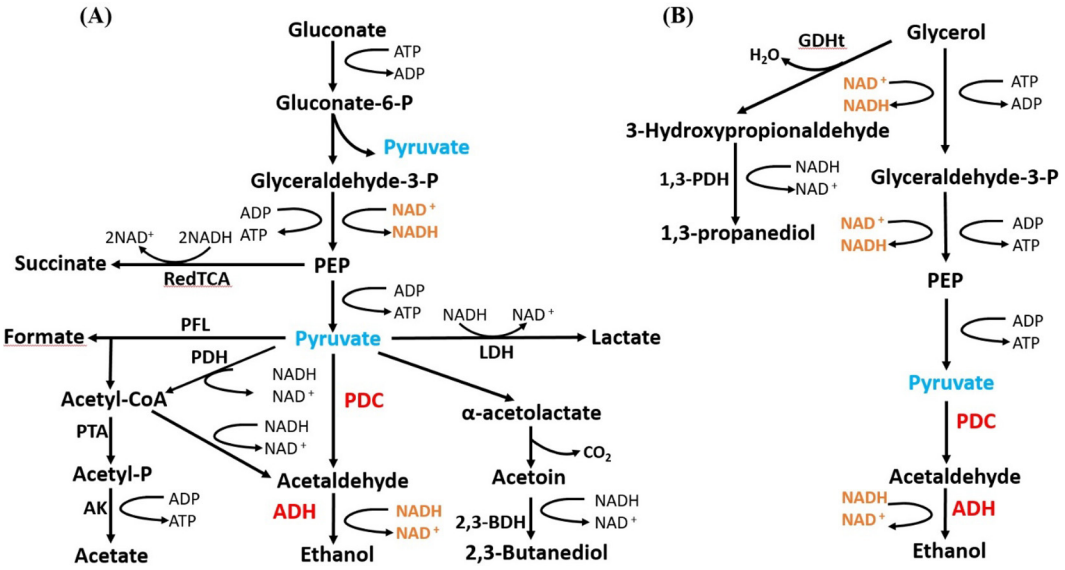

FIG 1 (A) Central metabolic pathways of gluconate metabolism in *K. oxytoca* BW21 under anaerobic conditions. RedTCA, reductive tricarboxylic acid cycle; PFL, pyruvate formate lyase; PTA, acetyl phosphotransferase; AK, acetate kinase; PDH, pyruvate dehydrogenase; LDH, lactate dehydrogenase; 2,3-BDH, 2,3-butanediol dehydrogenase; PDC, pyruvate decarboxylase (imported from *Zymomonas mobilis*); ADH, alcohol dehydrogenase (imported from *Zymomonas mobilis*). (B) Central metabolic pathways of glycerol metabolism in *K. oxytoca* BW21 under anaerobic conditions. GDHt, glycerol dehydratase; 1,3-PDH, 1,3-propanediol dehydrogenase; PEP, phosphoenolpyruvate.

two NADH are consumed to produce metabolites such as succinate, lactate, and ethanol through reduction reactions. In the reductive pathway, glycerol is first converted to 3-hydroxypropionaldehyde (3-HPA) by glycerol dehydratase (DhaB) and then reduced to 1,3-propanediol by NADH-dependent 1,3-propanediol oxidoreductase (DhaT) or NADPH-dependent aldehyde reductase/alcohol dehydrogenase. The overall reductive pathway consumes NADH. When glycerol is converted to pyruvate in *K. oxytoca* P2, two NADHs are generated (Fig. 1B and 2); the subsequent conversion of one

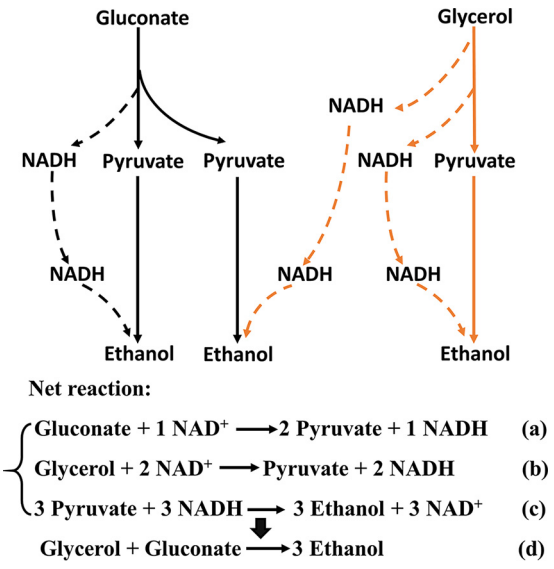

FIG 2 Metabolic pathways of engineered strain *K. oxytoca* involved in fermentative coutilization of gluconate and glycerol. Orange lines represent the glycerol utilization pathway, and the black lines represent the gluconate utilization pathway. (a) Gluconate to pyruvate via the ED pathway; (b) glycerol to pyruvate via an oxidative pathway; (c) pyruvate to ethanol; (d) coutilization of gluconate and glycerol to ethanol.

pyruvate to ethanol consumes only one NADH via the PDC pathway (24). When gluconate is converted to two pyruvates, only one NADH is produced, whereas the subsequent conversion of the pyruvate to two ethanols requires two NADHs via the PDC pathway. If glycerol and gluconate are utilized simultaneously, the excess NADH generated during pyruvate production in the glycerol oxidative process could be used for ethanol production from the extra pyruvate generated by the ED pathway, and homoethanol production is potentially achievable.

K. oxytoca naturally utilizes a wide variety of carbon sources, including glucose, xylose, cellobiose, cellotriose (25), xylobiose (26), and glycerol (27). *K. oxytoca* P2 contains three primary pathways that compete with the *Zymomonas mobilis* ethanol pathway for pyruvate under anaerobic conditions (Fig. 1): one pathway produces acetate through acetyl coenzyme A via *pfl*, another produces lactic acid by lactate dehydrogenase (*ldh*), and a third produces 2,3-butanediol through *budA* (α-acetolactate decarboxylase) and *budB* (α-acetolactate synthase). In addition, a fourth includes the production of succinate via fumarase reductase (*frd*) pathway. The 2,3-butanediol production pathway genes in *K. oxytoca* P2 were deleted, yielding the strain *K. oxytoca* BW21 (28). The major competing pathways for the conversion of glycerol to pyruvate are the production of 1,3-propanediol via glycerol dehydratase and the 1,3-propanediol oxidoreductase pathway (Fig. 1B).

In this study, we investigate improving ethanol yields from gluconate and glycerol using a cosubstrate fermentation strategy. Moreover, the possibility of further improving ethanol yields was attempted by deleting by-product formation pathways in strain BW21, resulting in *K. oxytoca* WT26. Lastly, the fed-batch culture approach was used to maximize the benefit of the redox recycling between the two substrates' utilization pathways and to achieve high ethanol yield.

RESULTS

Ethanol production from gluconate, glycerol, and their mixture. Strain *K. oxytoca* BW21 was used as an ethanologen to produce ethanol using gluconate, glycerol, and their mixture as the substrate. As shown in Fig. 3A, when 48.3 mM glycerol was used as the carbon source, about 30.8 mM ethanol was produced. The main by-products were 1,3-propanediol (8.7 mM) and acetate (3.6 mM). All glycerol got consumed within 8 h. When 52.83 mM gluconate was used as the carbon source, ethanol (65.85 mM) and acetate (25.06 mM) were produced as the two major products, as shown in Fig. 3B. Other by-products included lactate (1.81 mM), succinate (1.49 mM), 1,3-propanediol (0.12 mM), and 2,3-butanediol (0.45 mM). All gluconate was consumed in 8 h.

When gluconate glycerol mixture was used as the carbon source, BW21 simultaneously utilized gluconate and glycerol and produced 121.06 mM ethanol (Fig. 3C). The ethanol produced from the gluconate and glycerol mixture (yield of 78.7%) was much higher than the total produced from gluconate (yield of 62.3%) or glycerol (yield of 63.8%) separately (Table 1). Acetate and 1,3-propanediol produced during the cofermentation were substantially lower than when gluconate or glycerol was used alone.

However, when gluconate and glycerol were cofermented, the rate of gluconate utilization during the cofermentation was slightly faster than when it was used alone. All of the gluconate was consumed within 6 h. When the gluconate was depleted, about one-third of the glycerol (16 mM) remained unconverted. Moreover, the glycerol utilization rate was even lower after gluconate was depleted than when gluconate was present.

Construction and characterization of mutant strains on gluconate and glycerol mixture. Although the cofermentation strategy increased the ethanol yield and decreased the production of by-products, we sought to further improve ethanol yield by deleting the competing pathways in the strain BW21. The competing pathway genes we deleted included the *pta* gene, which encodes the phosphate acetyltransferase for acetate synthesis; the *frdABCD* genes, which encode the fumarate reductase enzyme complex for succinate production; the *ldh* gene, which encodes the lactate dehydro-

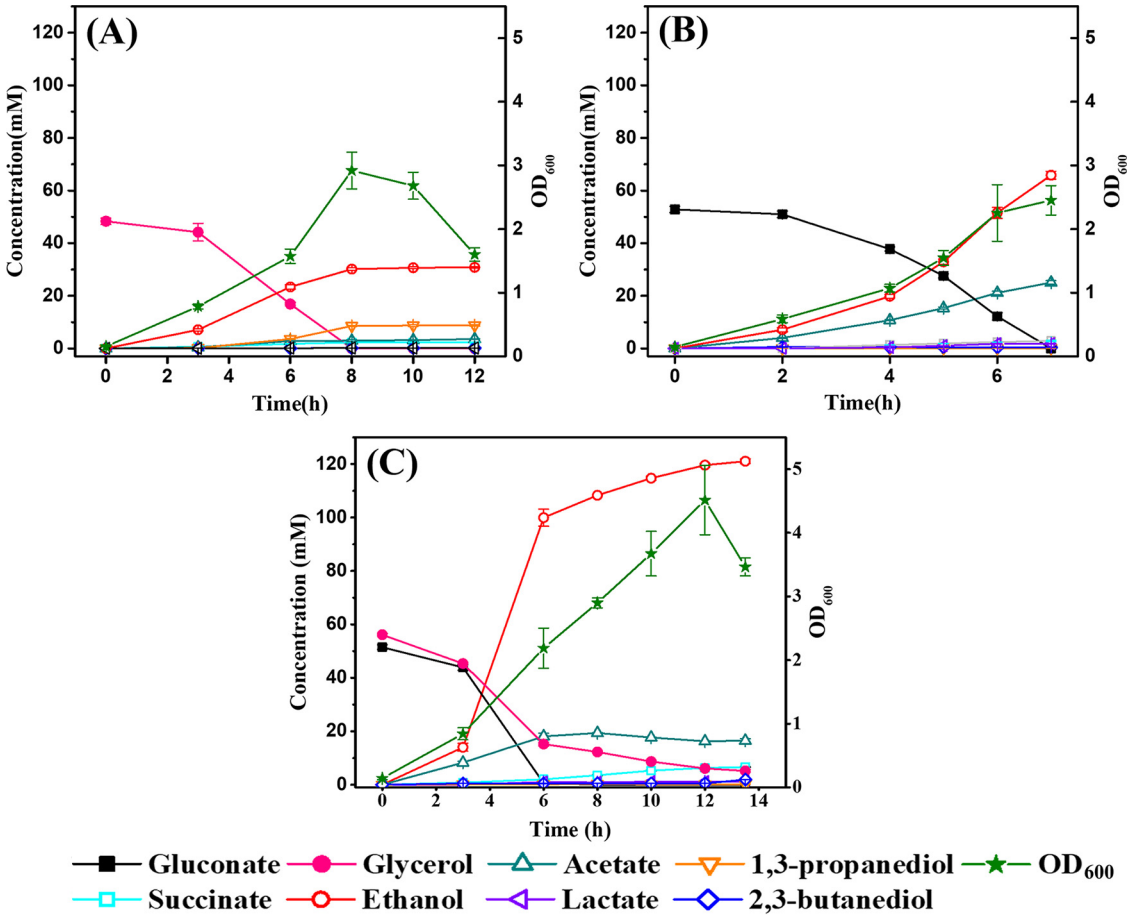

FIG 3 Fermentation characteristics of *K. oxytoca* BW21 on different carbon sources: glycerol (A), gluconate (B), and both gluconate and glycerol (C). The experiments were conducted in triplicate. Error bars indicate the standard deviations of sample replicates.

genase for lactate production; and the *pduC* gene, which encodes the glycerol dehydratase large subunit. We also chose to delete the *pflA* gene, which encodes pyruvate formate lyase (PFL) activating enzyme I, instead of *pflB*, which encodes PFL, to further block carbon flow toward acetate production. Strain BW21 showed substantial PFL activity because it produced substantial amounts of hydrogen when grown on glucose as the carbon source (data not shown). *E. coli* KO11, which had the PET operon inserted in *pflB* locus in the chromosome, likely originated by a single-crossover event, maintaining a functional copy of *pflB* downstream of the PET operon (27). Since strain *K. oxytoca* P2, the parent strain of *K. oxytoca* BW21, was constructed using the same approach by the same lab, it is likely that a functional *pflB* gene was also maintained by a single recombination event. For this reason, we chose to delete the *pflA* gene, which is present in a single copy and located downstream of the PET operon, to avoid the risk of altering or removing the PET operon. The deletion of the *pflA* was reported

TABLE 1 Metabolites produced by strain BW21 using different carbon sources[a]

Carbon source	Mean concn (mM) of glycerol consumed	Mean concn (mM) of gluconate consumed	Mean product concn (mM)						Mean ethanol yield (%)	Mean carbon recovery (%)
			Succinate	Lactate	Acetate	1,3-Propanediol	2,3-Butanediol	Ethanol		
Glycerol	48.3 ± 1.2		1.2 ± 0.2^{A}	0.2 ± 0.0^{A}	3.6 ± 0.1^{A}	8.7 ± 0.3^{A}	0.2 ± 0.1^{A}	30.8 ± 0.2^{A}	63.8 ± 1.6^{A}	93.0 ± 3.3^{A}
Gluconate		52.8 ± 1.2	1.5 ± 0.3^{A}	1.8 ± 0.1^{B}	25.1 ± 0.7^{B}	0.1 ± 0.1^{B}	0.5 ± 0.0^{B}	65.9 ± 1.5^{B}	62.3 ± 2.5^{B}	89.9 ± 3.3^{A}
Glycerol and gluconate	51.0 ± 0.4	51.5 ± 0.9	3.4 ± 0.1^{B}	1.2 ± 0.1^{C}	16.5 ± 0.7^{C}	0.2 ± 0.0^{B}	1.9 ± 0.0^{C}	121.1 ± 0.9^{C}	78.7 ± 1.3^{C}	93.7 ± 1.7^{B}

[a]All data are means from three sample replicates ± the standard deviations. Values with different superscript letters are statistically different ($P < 0.05$) according to Tukey's test.

TABLE 2 Characterization of metabolites production by mutant strains using gluconate and glycerol mixture as cosubstrates[a]

Strain	Glycerol consumed (mM)	Gluconate consumed (mM)	Products (mM)						Ethanol yield (%)	Carbon recovery (%)
			Succinate	Lactate	Acetate	1,3-Propanediol	2,3-Butanediol	Ethanol		
BW21	38.4 ± 1.1	45.3 ± 0.2	3.4 ± 0.0^{A}	0.5 ± 0.0^{A}	16.9 ± 0.2^{A}	0.5 ± 0.0^{A}	0.5 ± 0.0^{A}	103.6 ± 1.3^{A}	80.3 ± 1.3^{A}	97.2 ± 1.6
WT22	30.8 ± 1.5	45.1 ± 0.1	5.8 ± 0.0^{B}	2.3 ± 0.1^{B}	2.1 ± 0.1^{B}	0.4 ± 0.0^{B}	2.2 ± 0.0^{B}	99.4 ± 0.5^{B}	82.3 ± 1.2^{A}	94.5 ± 1.4
WT23	31.1 ± 0.2	45.1 ± 0.1	3.3 ± 0.0^{C}	4.2 ± 0.1^{C}	2.1 ± 0.2^{B}	0.2 ± 0.0^{C}	2.2 ± 0.1^{B}	100.1 ± 0.3^{B}	82.5 ± 0.4^{A}	94.2 ± 0.7
WT24	29.9 ± 0.8	45.0 ± 0.3	3.7 ± 0.0^{D}	0	1.9 ± 0.1^{B}	0.4 ± 0.0^{B}	2.3 ± 0.0^{B}	103.0 ± 0.5^{B}	85.9 ± 1.1^{B}	94.7 ± 1.2
WT25	26.5 ± 0.1	45.1 ± 0.0	3.3 ± 0.0^{C}	0	1.4 ± 0.0^{C}	0.4 ± 0.0^{B}	2.3 ± 0.0^{B}	101.9 ± 0.3^{B}	87.3 ± 0.2^{B}	95.6 ± 0.3
WT26	26.3 ± 2.5	47.7 ± 1.6	2.8 ± 0.1^{E}	0	0.7 ± 0.1^{D}	0.2 ± 0.0^{C}	1.8 ± 0.1^{C}	108.6 ± 0.6^{C}	89.3 ± 4.2^{B}	95.2 ± 4.5

[a]All data are means from three sample replicates ± the standard deviations. The initial gluconate and glycerol concentrations were 45 and 40 mM. Values with different superscripts are statistically different ($P < 0.05$) according to Tukey's test.

to cause a loss in intracellular PFL activity similar to the extent of loss caused by the deletion of *pflB* (29). Strain BW26 in which *pflA* has been deleted produced a negligible amount of hydrogen when the strain was grown on glucose.

Strain BW21 and derived mutant strains were characterized for ethanol and by-products production on gluconate (45 mM) and glycerol mixture (40 mM) as the carbon source. The results are shown in Table 2. The final construct strain, WT26, yielded significantly higher amounts of ethanol than did strain BW21.

Ethanol production from gluconate, glycerol, and their mixture by strain WT26. Strain BW26 was characterized for ethanol and coproduct production using gluconate, glycerol, and their mixture as the carbon source. As shown in Fig. 4 and Table 3, WT26 consumed about 52 mM gluconate in about 12 h, which is slower than

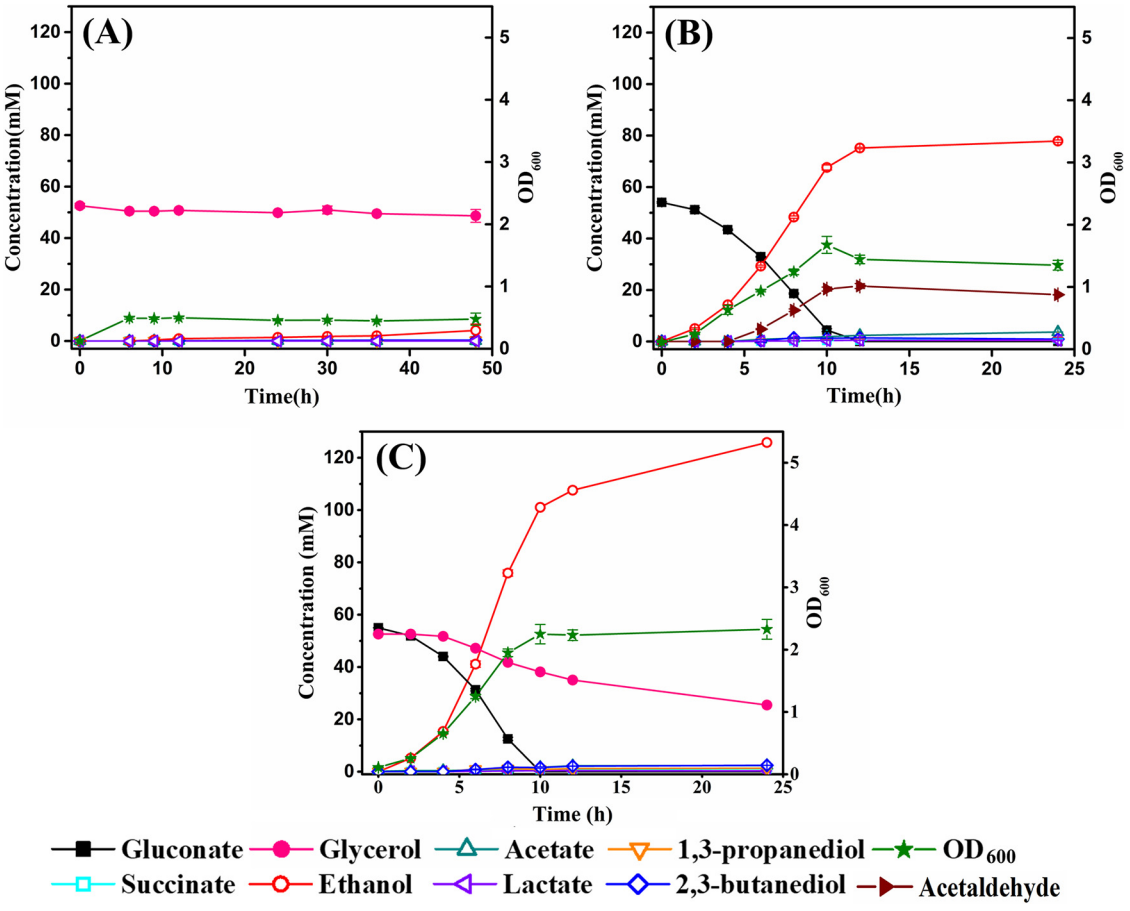

FIG 4 Fermentation characteristics of *K. oxytoca* WT26 on different carbon sources: glycerol (A), gluconate (B), and both gluconate and glycerol (C). The experiments were conducted in triplicate. Error bars indicate the standard deviations of sample replicates.

TABLE 3 Metabolites produced by the strain WT 26 using different carbon sources[a]

Carbon source	Glycerol consumed (mM)	Gluconate consumed (mM)	Product concn (mM)							Ethanol yield (%)	Carbon recovery (%)
			Succinate	Lactate	Acetate	1,3-Propanediol	2,3-Butanediol	Acetaldehyde	Ethanol		
Gluconate		54.1 ± 0.3	1.0 ± 0.1^{A}	0.5 ± 0.2^{A}	3.7 ± 0.0^{A}	1.3 ± 0.2^{A}	0.9 ± 0.3^{A}	18.2 ± 0.1^{A}	77.9 ± 0.5^{A}	72.0 ± 0.6^{A}	96.5 ± 1.7
Glycerol and gluconate	27.1 ± 0.2	55.0 ± 0.1	1.1 ± 0.1^{A}	0.3 ± 0.0^{A}	2.4 ± 0.0^{B}	1.3 ± 0.0^{A}	2.4 ± 0.0^{B}	0^{B}	125.8 ± 0.3^{B}	91.8 ± 0.4^{B}	99.0 ± 0.4

[a]All data are means ± the standard deviations from three sample replicates. Values with different superscripts are statistically different ($P < 0.05$) according to Tukey's test.

that for BW21. The ethanol yield increased by almost 10% compared to BW21, from 62.3 to 72%. Strain WT26 could barely use glycerol. When gluconate and glycerol were coutilized in batch culture, gluconate and glycerol were used simultaneously. However, gluconate was used much faster than glycerol. The average rate of gluconate consumption during the first 10 h was about 5.5 mM/h, while the average rate of glycerol consumption was only 1.5 mM/h. By the time gluconate was completely consumed at 10 h, about 38 mM glycerol had remained unconverted. Glycerol continued to be consumed after gluconate was depleted until about 24 h (Fig. 4), and then glycerol consumption stopped (data not shown). About half of the glycerol remained unutilized after 24 h. The overall yield of ethanol from the consumed gluconate and glycerol was about 91.8%.

Production of ethanol from gluconate and glycerol by strain WT26 using a continuous fed-batch mode. Because gluconate was used faster than glycerol in batch fermentation, the benefits of redox balance could not be fully realized. We conducted the fermentation in a fed-batch mode to solve this problem. The fed-batch process was started in the reactor by inoculating WT26 into LB medium containing 102 mM glycerol. LB medium containing gluconate (95 mM) was then continuously fed to the reactor to provide the cosubstrate. The gluconate feeding schedule and the rates of gluconate and glycerol consumption are shown in Table 4. During the process of fed-batch fermentation, gluconate was maintained at low concentrations (<0.05 mM), and glycerol was consumed at the end of the fermentation. As shown in Fig. 5, about 154 mM ethanol was produced. The major detectable by-products was acetate (3.4 mM). The total amount of glycerol consumed was 31.5 mmol, and the total amount of gluconate consumed was about 28.3 mmol. The overall yield of ethanol was about 97.2%.

DISCUSSION

K. oxytoca is one of a few bacterial strains that are capable of utilizing glycerol as a carbon source fermentatively. Our study demonstrated for the first time that *K. oxytoca* could also use gluconate as the substrate for high-yield ethanol production. The ethanol yield achieved by strain BW21 (62%) was comparable to that achieved by a sister strain, *E. coli* KO11 (65%) (14).

We hypothesized that coutilization of gluconate and glycerol could increase the ethanol yield by recycling the excess NADH produced during the glycerol oxidative pathway used for ethanol production using pyruvate generated during gluconate metabolism. Our experimental results showed a significant increase in ethanol yield to about 78.8% and a substantial decrease in by-product formation (acetate and 1,3-

TABLE 4 Gluconate feeding schedule and rates of gluconate and glycerol consumption during fed-batch culture

Time span (h)	Gluconate feeding rate (ml/h)	Consumption rate (mmol/h)	
		Glycerol	Gluconate
0–4	1.3	0.16	1.38
4–12.5	10.6	1.67	1.17
12.5–15.5	16.7	2.12	1.85
15.5–21	20.0	1.89	2.22

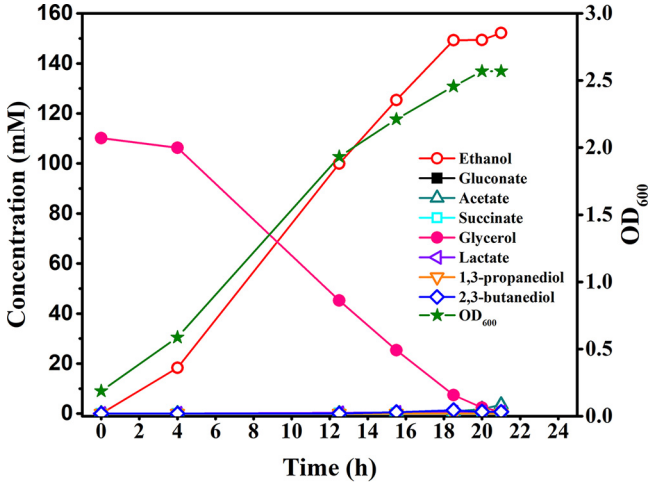

FIG 5 Production of ethanol and coproducts by *K. oxytoca* WT26 from gluconate and glycerol in continuous fed-batch fermentation. The experiments were repeated three times.

propanediol). Gluconate and glycerol were used simultaneously. While we observed no obvious sign of carbon catabolite repression, there was a significant discrepancy between the respective utilization rates of gluconate and glycerol. Much of the glycerol remained unconverted once the gluconate had been completely consumed.

The actual ratio of the consumed gluconate versus consumed glycerol was about 1:0.67, which deviated from 1:1. About one-third of glycerol was utilized uncoupled with gluconate. Both of these two factors led to a lower ethanol yield. Moreover, glycerol utilization was even slower when gluconate was depleted than when gluconate was present. The reason behind this finding is still unclear. Noteworthy, the cofermentations of glucose plus gluconate and glycerol led to catabolite repression of glycerol consumption (data not shown), which is a problem that needs to be addressed in future studies.

Deleting the competing pathways led to a gradual increase in ethanol yield over the gluconate glycerol mixture. The mutant WT26 achieved the highest ethanol yield in batch fermentation using gluconate and glycerol mixture as the carbon source. However, strain WT26 could no longer grow fermentatively on glycerol as the sole carbon source. Specifically, the deletion of 1,3-propanediol production pathway in WT26 eliminated the route for the extra NADH generated during pyruvate production to be reoxidized back to NAD^+. The redox imbalance led to the inability of the strain to use glycerol as the sole carbon source. However, strain WT26 successfully consumed 52 mM gluconate in 12 h. Redox balance was maintained by the production of a substantial amount of acetaldehyde (17 mM) as the oxidized product after the acetate production pathway was deleted. The ethanol yield of 72% was substantially higher than that of strain BW21 (62%) and was close to the theoretical maximum that could be achieved using gluconate as the substrate (75%). When the gluconate and glycerol mixture was used as the carbon source, the yield of ethanol from consumed gluconate and glycerol increased as expected, the discrepancy between the gluconate and glycerol utilization rates increased also.

Fed-batch fermentation is an effective strategy to achieve better fermentation performance by controlled addition of substrates or nutrients. It has been used to overcome substrate inhibition and carbon catabolite repression, to achieve high cell density fermentation, to cultivate of auxotrophic mutants, and to decrease the viscosity of fermentation broth (30). In this study, we used the fed-batch approach to match the rate of metabolism of a quickly utilized substrate (gluconate) to that of a slowly utilized one (glycerol) for the purpose of maximizing the benefit of redox recycling between their respective metabolic pathways. The total amount of glycerol consumed was controlled to match that of gluconate. As a result, close to the theoretical ethanol yield was achieved from these two substrates through the controlled continuous fed batch

TABLE 5 Strains and plasmids used in this study

Strain or plasmid	Description[a]	Source or reference
K. oxytoca strains		
BW21	M5A1, *pflB*::(Km^r *pdc adhB*) *cat*, Δ*budAB*; Amp^r	28
WT22	BW21 Δ*pta*; Amp^r	This study
WT23	BW21 Δ*pta* Δ*frd*; Amp^r	This study
WT24	BW21 Δ*pta* Δ*frd* Δ*ldh*; Amp^r	This study
WT25	BW21 Δ*pta* Δ*frd* Δ*ldh* Δ*pflA*; Amp^r	This study
WT26	BW21 Δ*pta* Δ*frd* Δ*ldh* Δ*pflA* Δ*pduC*; Amp^r	This study
Plasmids		
pKD4	FRT-Km^r-FRT	36
pSIJ8	pkD46, *rhaRS*-p*rha*-*FLP*; Amp^r	35
pSIJ8-*tet*	pkD46, *rhaRS*-p*rha*-*FLP*; Tet^r	This study

[a]Amp^r, ampicillin resistance; Tet^r, tetracycline resistance; Km^r, kanamycin resistance; *rhaRS*-p*rha*, rhamnose-responsive regulators and corresponding promoter.

fermentation. The problem of incomplete conversion of glycerol in batch culture was also successfully resolved.

There are many successful examples in the literature reporting cosubstrate fermentation to achieve better fermentation performance and to increase product yields. Adding sugars, mannitol, or hemicellulose hydrolysate as a cosubstrate to glycerol can significantly increase the yield of 1,3-propanediol production (31–33). Beet molasses added as a cosubstrate promoted 2,3-butanediol production from biodiesel derived glycerol (34). Although these cosubstrates contributed to the production of cell biomass and regeneration of reducing power, the cosubstrates themselves were not converted into products and represent an additional processing cost. Our study provides another successful example of yield increase by cosubstrate fermentation. Cofermentation of glycerol and gluconate is unique in that cosubstrates are mutually beneficial, and both substrates contributed to yield increases in cofermentation. Through pathway engineering and adoption of the fed-batch culture, we achieved high-yield homoethanol production that usually can be achieved by using glucose as the substrate. The same strategy is expected to be able to achieve homofermentative production of other products such as the 2,3-butanediol, lactate, which can be typically achieved using glucose as the substrate, using inexpensive biodiesel derived glycerol and biomass-derived gluconate as the cosubstrates.

MATERIALS AND METHODS

Strains, plasmids, and materials. The *K. oxytoca* BW21 strain used in this study was kindly provided by the Ingram group at the University of Florida (28). The strains and plasmids used in this study are summarized in Table 5. Restriction and DNA-modifying enzymes were purchased from New England Biolabs (Ipswich, MA). The Miniprep and DNA gel extraction kits were purchased from Zymo Research (Irvine, CA). The primers were synthesized by Integrated DNA Technologies (Coralville, IA). All chemicals used in the fermentation study, as well as the high-performance liquid chromatography (HPLC) standards, were purchased from Sigma-Aldrich (St. Louis, MO).

Construction of the *K. oxytoca* mutant strains. The knockout strains were constructed using a one-step disruption protocol established by Jensen et al. (35) using a plasmid containing a lambda red recombineering gene under the arabinose-inducible promoter, a flippase recombinase (FLP) gene under a rhamnose-inducible promoter, a temperature-sensitive replication origin, and an antibiotic-resistant selection marker. Plasmid pSIJ8 with an ampicillin resistance selection marker was ordered from Addgene.com. Given that *K. oxytoca* BW21 is naturally resistant to ampicillin, we replaced the ampicillin-resistant gene with a tetracycline-resistant gene in the plasmid pSIJ8, resulting in plasmid pSIJ8-*tet*. *K. oxytoca* BW21 was transformed with the plasmid pSIJ8-*tet*. Knock-in-cassette PCR products were constructed using plasmid pKD4 (35) (which contains an FRT-flanked kanamycin-resistant gene) as a template using primers with 50- to 100-bp extensions that are homologous to the regions upstream and downstream of the target genes. The amplified FRT-Kan^r-FRT cassettes were introduced by electroporation in the strains harboring pSIJ8-*tet* and recombined in the target chromosomal sites by lambda red-based recombineering.

The removal of antibiotic resistance markers was achieved by inducing FLP with 15 mM L-rhamnose (35). After confirming the integration of cassettes and the removal of resistant markers by diagnostic PCR, pSIJ8-*tet* plasmids were cured from the cells by growing them at 37°C. All of the knockout mutants were double checked by diagnostic PCR and antibiotic resistance testing. The primers used for strain construction and verification are summarized in Table 6.

TABLE 6 Primers used in strain construction and verification

Gene	Primer sequence (5′–3′)	
	Forward	Reverse
Primers for amplifying the FRT flanked Kan^r gene from plasmid pKD4		
pta	GAAGGCGGCGACGCAGAGAATCCCCAGGAGCGTACGAGTAG TACGTGTAGGCTGGAGCTGCTTC	ACGGCGCATGATATTGTCTCGGCGGCGCGAGAGCTGCTGTA AATCATATGAATATCCTCCTTAG
frd	TGACCCTACGGCTATACTCCTTACCTATAAAGGAGCAGTGAT ACGTTTTCGCCAGCCCGGCGGCGAAATAAACAAAAACT GGAGGAATGTCCGATTGTGTAGGCTGGAGCT	ATGCGGCAGAATTTTCTTGAGAAGCGAGCCCGGTAACAGA ATGCTACCGGGCTTATTGCAGCTGAGGTAAAAGCCGC GTACGGCTATCCGTTGGGAATTAGCCATGGTCCA
ldh	TCCAGCCGCCAGCAGCGCAGCAAACTTGTTCATTTAGTCT CCATAACTATTTATCGTATTAATAAATGAAGGGAA TTGTACGATTGTGTAGGCTGGAGCT	CAACTTTTAATGAAACGTATTGTCTTTTAAAGAGATTTCTTA ACTCTACGATATGCTCTAGAATTAATACTATAACC TGCTGGGAATTAGCCATGGTCCA
pflA	ACATAGGCCCCGGATGGGCCAAATTCGGAGATATCACC GCAATGGTGTAGGCTGGAGCTGCTTC	TGGGGAAACACCCGGTAGCGCAATCTCTACCGGGTGGGGA TTTTAGGAACACTTAACGGCTGACATG
pduC	CTTCATTTCGCTAAGCACGTCTTCAATTATCTGGCGCA GCAATTTTTCATTAATTTCCATTTCTCACC CCCGATTGTGTAGGCTGGAGCT	TCTTGCGACCCTCGGTGCTGAACCGAAAAACGATCGCCCG TCCTACATCTGATACCCACGAGGCTGATTC TGGGAATTAGCCATGGTCCA
Primers used for diagnostic PCR (designed based on the sequence of the gene ORF)		
pta	GAACCGCTGAAGATGAGCCAC	CTACGCGCACAGAAGGTGAC
frd	GCGATGTGGTCTATCTCGATCT	CCAGTTCTCGTTACCTTCCTGA
ldh	GACGCAGGTTGTCTAGGGTG	CTGGATATGGGCGTGGAGTATG
pflA	GTACTGTTCCAGAATGCCCTTCAC	TATCAGGACATAGCGTTGGCTACC
pduC	GGCTTCAACTTCTTCATCGGTA	CAGACCTTTACCCACTCCGATA
Kan^r	TTGAACAAGATGGATTGCACGCAG	ACTCGTCAAGAAGGCGATAGAAGG
Primers used for diagnostic PCR (designed based on sequences upstream and downstream of the specific gene)		
pta	TCGGCAAGTCTGGCTTCATC	GTTTACCCTGAAGCCCATCGAC
frd	GAAACAAGGGCACGAAGTCTAC	AGGTACCAAGATAGCGTTGAGC
ldh	TGTTCCGCTCTGGTTGACAC	TTTACTCTGGCCGCTGGTC
pflA	TCGATTGAGAGCGTGGCGAATAAG	GACTCGACATAGCCTTTGAGCTG
pduC	GACGGCGATAATCACTTCGT	GTGATTTCCGGCGACTCC

Media and cultivation conditions. Cells were precultured from a single colony in 5 ml of LB liquid medium inside a 15-ml centrifuge tube at 30°C for 8 h. One milliliter of the preculture was transferred to a 200-ml seed serum bottle containing 100 ml of LB, followed by incubation for 12 h at 30°C and 200 rpm. The seed culture was then added to fermentation serum bottles containing 100 ml of LB with various concentrations of carbon sources (gluconate, glycerol, or both). Bottles were pH adjusted to 7.0 with sodium hydroxide and purged with argon for 1 min to create anaerobic conditions for fermentation. All fermentation experiments were run on a rotary shaker at 30°C and 200 rpm. Samples were taken at various time intervals to analyze the concentrations of metabolites and cells in the fermentation broth.

Fed-batch experiments were conducted in a Bio-Flo115 reactor with a working volume of 600 ml. The reactor contained 270 ml of 101 mM glycerol in LB medium. The fermentation started by inoculating the strain WT26 at an initial optical density of 0.15. LB medium containing 95 mM gluconate was continuously fed to the reactor over a period of 21 h. Argon gas was sparged to ensure the anaerobic environment. Samples were taken and analyzed at different time intervals.

Analysis methods. Cell growth was determined by measuring the optical density at 600 nm. The concentrations of glucose, gluconate, glycerol, 1,3-propanediol, lactate, ethanol, acetate, formate, 2,3-butanediol, and succinate were determined by HPLC with an IC Sep Ion-300 column (Transgenomic, San Jose, CA), a refraction index detector, and a photodiode array detector. The mobile phase used was 5.0 mM sulfuric acid with a flow rate of 0.5 ml/min (14).

Calculations of the yields. The yields of the products in batch fermentation were calculated according to the following equation:

$$\text{product yield} = \frac{\text{product (mM)}}{\text{gluconate consumed (mM)} \times 2 + \text{glycerol consumed (mM)}} \times 100\%.$$

The carbon recovery was calculated according to the following equation:

$$\text{carbon recovery} = \frac{\Sigma\ \text{C}_2 \text{ and C}_3 \text{ metabolites (mM)} + \text{2,3-butanediol (mM)} \times 2 + \text{ succinate (mM)}}{\text{gluconate consumed (mM)} \times 2 + \text{glycerol consumed (mM)}} \times 100\%.$$

C_2 metabolites include acetate, acetaldehyde, and ethanol. C_3 metabolites include 1,3-propanediol and lactate.

The ethanol yield achieved in the fed-batch culture was calculated using the following equation:

$$\text{ethanol yield} = \frac{\left(\sum_{j=1}^{n} E_j V_j + V_f E_f\right) \times 100\%}{\left(V_i G_i - V_f G_f - \sum_{j=1}^{n} G_j V_j\right) + \left(VGa_i - VGa_f - \sum_{j=1}^{n} Ga_j V_j\right) \times 2},$$

where V_i is the initial reactor volume (ml), V_f is final reactor volume (ml), V is the total volume of LB medium containing gluconate fed to the reactor, V_j is the sample volume (ml), E_j is the ethanol concentration (mM) in the sample, G_j is the glycerol concentration (mM) in the sample, Ga_j is the gluconate concentration (mM) in the sample, Ga_i is the gluconate concentration (mM) in the fed bottle, Ga_f is the gluconate concentration (mM) in the reactor at the end of the fermentation, E_f is the ethanol concentration (mM) in the reactor at the end of the fermentation, and G_f is the glycerol concentration (mM) in the reactor at the end of the fermentation.

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