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# Citrate Synthase Overexpression of *Pseudomonas putida* Increases Succinate Production from Acetate in Microaerobic Cultivation

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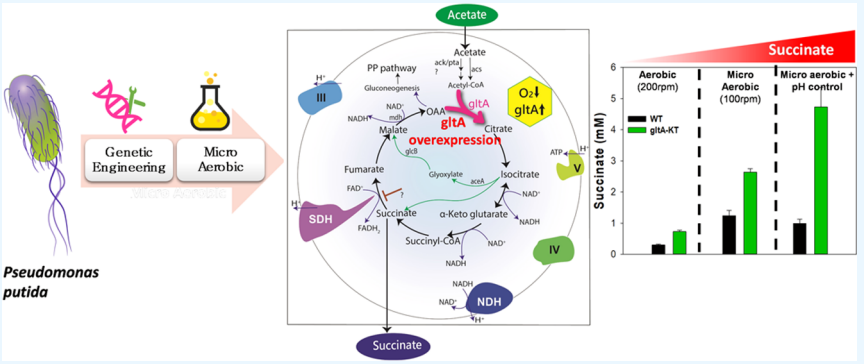


**ABSTRACT:** Acetate is an end-product of anaerobic biodegradation and one of the major metabolites of microbial fermentation and lingo-cellulosic hydrolysate. Recently, acetate has been highlighted as a feedstock to produce value-added chemicals. This study examined acetate conversion to succinate by citrate synthase (*gltA*)-overexpressed *Pseudomonas putida* under microaerobic conditions. The acetate metabolism is initiated with the gltA enzyme, which converts acetyl-CoA to citrate. *gltA*-overexpressing *P. putida* (*gltA*-KT) showed an ∼50% improvement in succinate production compared to the wild type. Under the optimal pH of 7.5, the accumulation of succinate (4.73 ± 0.6 mM in 36 h) was ∼400% higher than that of the wild type. Overall, *gltA* overexpression alone resulted in 9.5% of the maximum theoretical yield in a minimal medium with acetate as the sole carbon source. This result shows that citrate synthase is important in acetate conversion to succinate by *P. putida* under microaerobic conditions.

## 1. INTRODUCTION

The bioproduction of succinate from renewable carbon sources can replace conventional petrochemical-based refinery processes.[1] According to the US-DOE report, succinate is one of the top 12 platform chemicals that require the development of a synthesis process.[2] Succinate is a building block to produce numerous valuable intermediates and materials in the food (flavor), pharmaceutical, and chemical (surfactant, ion chelator, paint coating, and sealant) industries.[3] Succinate has recently been used as a feedstock for synthesizing tetrahydrofolate (THF), 1,4-butanediol, and maleic anhydride.[4,5] Previous research reported the bioproduction of succinate through the microbial anaerobic fermentation of glucose.[6] On the other hand, the use of glucose is unsustainable. Therefore, it is necessary to develop an alternative bioprocess using more renewable and sustainable carbon sources, such as xylose (from plant biomass),[7] glycerol,[6] and acetate.

Acetate ($CH_3COO^-$) is a simple two-carbon carboxylic acid produced mainly from organic chemical synthesis and microbial fermentation.[8] Chemical synthesis through the carbonylation of methanol or the oxidation of acetaldehyde provides approximately 75% of the industrial demand.[9] Few companies produce bioacetate through microbial fermentation (Biocorn products, Afyren, and Godavari Biorefineries). An acidic pretreatment of cellulose releases large quantities of acetate.[10] Microbial electrosynthesis (MES) has recently been used to generate acetate from $CO/CO_2$ using renewable electricity.[11−14] Acetate is an important intermediate to implementing biological carbon capture and utilization (CCU) processes in practical applications. Therefore, acetate, which is a nontraditional carbon feedstock for bioconversion, will be highlighted in industrial biotechnology because it is inexpensive and decoupled with the food supply chain.[15]

Acetate has the potential to serve as a carbon source for the bioconversion of various compounds, including fatty acids, PHB, 3-hydroxy propionic acid, tyrosine, phloroglucinol, glutamate, mevalonic acid, glycolate, and succinate.[16] On the

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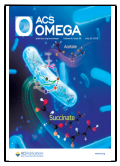

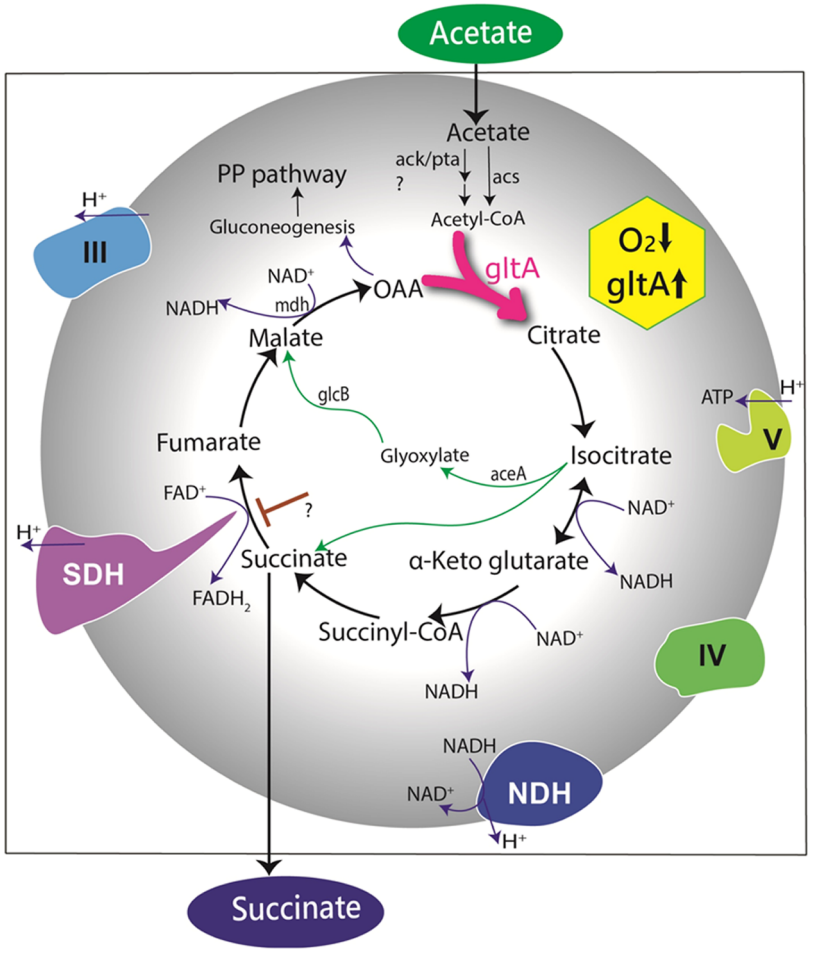


**Figure 1.** Schematic diagram of the metabolic pathway of succinate production from acetate in citrate synthase (*gltA*) gene-overexpressed *P. putida* (*gltA*-KT) under microaerobic conditions: acs, acetyl-CoA synthetase; ackA, acetate kinase; pta, phosphotransacetylase; gltA, citrate synthase; SDH, succinate dehydrogenase ABCD; mdh, malate dehydrogenase; aceA, isocitrate lyase; NAD, NADH dehydrogenase; III & IV electron transport chain complexes; V, ATP synthase.

other hand, most studies reported that acetate accumulation is frequently toxic to microorganisms at high concentrations because it is a weak organic acid with a relatively small molecule size that can penetrate the cell membrane. Therefore, selecting an appropriate host strain is essential for acetate bioconversion.

*Pseudomonas putida* KT2440 exhibits innate tolerance to toxic substances, including ethanol and aromatic chemicals, making it a valuable host strain for recombination in bioconversion processes. In addition, it possesses versatile carbon metabolic pathways and operates on minimal maintenance energy, further enhancing its suitability as a host strain.[17] Various genetic engineering tools are being developed for *P. putida* KT2440 through genome analysis.[18] Previous studies focused on the synthesis of natural and heterologous bioproducts, such as biofuels, lipids (rhamnolipids), polymers (polyhydroxyalkanoate), organic acids (adipic acid), and indigoidine.[19] In particular, succinate production from glucose and glycerol under nitrogen-limited conditions was observed in *P. putida*.[20,21] With the characteristics listed above, this strain could be a potential host for succinate production from acetate.

This study examined the overexpression of citrate synthase (*gltA*), a bottleneck enzyme for acetate assimilation into the TCA cycle of *P. putida*. The effects of such recombination on cell growth, acetate consumption, and succinate production were examined using acetate as a sole carbon source. The results highlight the significance of *gltA* on acetate assimilation and simultaneous succinate production of *P. putida* (Figure 1). To the authors' knowledge, this is the first study of succinate production from acetate, particularly in a minimal medium and under microaerobic conditions using *P. putida* with overexpression of *gltA*.

## 2. MATERIALS AND METHODS

**2.1. Cultivation Method and Medium.** The seed culture was prepared by inoculating (0.05 $OD_{600}$) aerobically grown preculture of wild-type and engineered strains of *P. putida* (30 °C and 200 rpm overnight) in an LB broth (50 mL) in a 250 mL flask. The seed culture was harvested at 5000 rpm, 10 min, and 4 °C after 12 h of cultivation. The cell pellet was washed twice with a minimal medium at 5000 rpm for 10 min under 4 °C. Finally, the washed pellet was dissolved in a minimal medium (pH 7.2). The minimal medium with different acetate concentrations (50, 100, 200, and 300 mM) was inoculated with 0.05 initial optical density ($OD_{600}$) to examine the effects of acetate concentration on bacterial growth. The effects of dissolved oxygen on acetate uptake and succinate production were examined by varying the agitation speeds. *P. putida* was cultured in a 250 mL flask with a 50 mL working volume under


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the following conditions: (i) aerobic cultivation at 200 rpm and (ii) microaerobic cultivation at 100 rpm.[22] In general, the agitation speed of a medium is related directly to the solubility of oxygen. As a relative term, a decrease in agitation speed is often considered to produce microaerobic conditions compared to the control with high-speed agitation in numerous studies.[23−26] Therefore, for this study, an agitation speed of 100 rpm was considered to represent a microaerobic condition. Streptomycin (100 μg/mL) was added to cultivate the *gltA* overexpressed engineered strain (*gltA*-KT). IPTG (0.5 mM) was added as an inducer to overexpress the *gltA* gene.

For the resting cell experiments, *P. putida* cells were harvested after 36 h of microaerobic cultivation in a minimal medium with 100 mM acetate. They were then resuspended in 50 mL of nitrogen-limited minimal medium with an initial $OD_{600}$ = 3.0 with various acetate concentrations (100−300 mM) in a 250 mL flask and cultivated for 48 h. Bacterial growth was restricted by reducing the nitrogen supply by omitting $(NH_4)_2SO_4$ from 1 g/L to 150 mg/L. The flasks were incubated at 100 rpm and 30 °C. The pH of the medium was measured at regular intervals and adjusted by 5N HCl to maintain a pH of ∼7.5 of the medium. The ATP supplementation experiment was conducted with varying ATP concentrations (0.5, 1, 2, and 3 mM) and at a pH of 7.5.

The cell was cultivated in a defined M9 medium consisting of the following components (per liter): $Na_2HPO_4{\cdot}12H_2O$ (15.12 g), $KH_2PO_4$ (3.0 g), NaCl (0.25 g), and $(NH_4)_2SO_4$ (1 g). This basic solution was autoclaved and supplemented with 0.49 g/L $MgSO_4$, and a trace element solution made of 6.0 mg/L $FeSO_4$ $7H_2O$, 2.7 mg/L $CaCO_3$, 2.0 mg/L $ZnSO_4$ $H_2O$, 1.16 mg/L $MnSO_4$ $H_2O$, 0.37 mg/L $CoSO_4$ $7H_2O$, 0.33 mg/L $CuSO_4$ $5H_2O$, and 0.08 mg/L $H_3BO_3$ (all filter-sterilized). The neutralized-acetate solution was added to the medium as a sole carbon source. All chemicals in LB and M9 media were purchased from BD Bioscience, Thermo Fisher Scientific, and Sigma. Luria−Bertani (LB) medium (per liter: tryptone, 10 g; yeast extract, 5 g; sodium chloride, 5 g) was used for primary culture cultivation, construction of plasmids, and plasmid amplification. During the cloning experiment, the *E. coli* DH5α strain was cultivated at 200 rpm and 37 °C and used as a plasmid amplification strain.

**2.2. Strain and Plasmids for the Overexpression of *gltA*.** The *P. putida* KT2440 (hereafter mentioned as *P. putida*) strain was engineered for the overexpression of *gltA*. Table 1 lists all of the strains used for cloning experiments and primers. The *gltA* expression vector was constructed by cloning the *gltA* gene in the IPTG-inducible plasmid pTD-NstrepHis (from Addgene, plasmid #45936). The *gltA* gene was amplified by PCR using the genomic DNA from *P. putida* as the template and oligonucleotide primers (Table 2). The resulting amplicons were ligated into the vector pTD-NstrepHis using the restriction sites EcoRI and BamHI.

The resulting plasmid was designated pTD_*gltA*. The *gltA* gene sequence of the pTD_*gltA* plasmid was confirmed by Sanger sequencing. The purchase of primers and sequencing were carried out through Macrogen (Seoul, Korea) and Bionics, respectively. The enzymes involved in gene cloning, ligation, and PCR were purchased from NEB (Ipswich, MA) and Bioneer (Daejeon, Korea).

**2.3. RNA Isolation and qRT-PCR.** The strains were cultivated in a minimal medium with acetate for 18 h ($OD_{600}$ ∼ 0.6−0.8) and harvested by centrifugation for 10 min at 7500 rpm. Total RNA isolation was conducted using a Bioneer RNA isolation kit according to the manufacturer's protocol. The RNA quality and concentration were checked by gel electrophoresis on 1% agarose gel and spectrophotometry (Nanodrop 2000, Thermo Scientific), and the ratio of absorbance at 260/280 nm was between 1.8 and 2.1. The cDNA was synthesized through a reverse transcription reaction using a commercial kit (iScript cDNA synthesis kit, Bio-Rad). Quantitative real-time PCR was performed using a qPCR master mix (SYBR green master mix, Takara, Japan). Each gene was amplified in triplicate. The $2^{-\Delta\Delta Ct}$ method was used to compare the relative gene transcription levels between the recombinant (*gltA*-KT) and the wild type. Table 2 lists the primers used for quantitative real-time PCR analysis. The gene *rpoD* was used as the internal standard.

**2.4. Analysis of Protein Expressions.** The protein expression of citrate synthase in the wild-type and *gltA*-KT strains, cultivated in 100 mM acetate at 100 rpm, was compared using SDS-PAGE. After 2 h incubation of the inoculated *P. putida* (see Section 2.1), IPTG (0.001, 0.01, 0.1, 0.5, 1, and 10 mM) was added to the medium. The cells were harvested after 18 h when the $OD_{600}$ reached ∼1.0 by centrifugation at 8000 rpm for 10 min. The harvested cells were washed with a 1× PBS buffer solution. The cells were dissolved and lysed in 1× SDS-PAGE sample buffer at 60 °C for 10 min. The soluble fractions of the cell lysate were collected by centrifugation at 18,000 rpm for 30 min at 4 °C and loaded into 15% sodium dodecyl sulfate-polyacrylamide gel. The electrophoresis of each sample ran at 80 V for 20 min and 120 V for 60 min. The relative protein expression levels upon induction were compared using a protein marker.

**2.5. Analytical Methods.** Cell growth was measured at an optical density of 600 nm ($OD_{600\,nm}$) using a UV-1200 spectrophotometer (Optizen POP, Keen Innovative Solutions, Daejeon, Korea). The acetate and succinate concentrations in the collected cell culture supernatant were quantified by HPLC (Agilent 1100 series Agilent Technologies, Santa Clara, CA), equipped with a 300 mm × 7.8 mm Aminex HPX-87H ion-exchange column (Bio-Rad) operating at 65 °C with a flow rate of 0.5 mL $min^{-1}$ of 2.5 mM $H_2SO_4$ using a refractive index detector (RID) for metabolite detection. For HPLC analyses, a 1 mL sample was taken and centrifuged at 12,000 rpm for 10 min. Subsequently, the supernatant, filtered through a nylon membrane filter (0.22 μm, Shanghai Instrument Consumables Co., Shanghai, China), was supplemented with streptomycin antibiotic. Organic acids in liquid samples were analyzed using TSQ Endura Liquid Chromatography/Mass spectrometer (LC-MS, Thermo Fisher Scientific). This LC-MS instrument, which was equipped with a heated electron spray ionization

**Table 1. Strains and Plasmids**

| | relevant characteristics | refs |
|---|---|---|
| | Strains | |
| *Pseudomonas putida* KT2440 | wild type | lab stock |
| *Escherichia coli* DH5α | strain for cloning and plasmid propagation | lab stock |
| *gltA*-KT | KT2440 harboring pTD-*gltA* | this study |
| | Plasmids | |
| pTD-NStrepHis | $Sm^r$, gene expression vector, IPTG-inducible | 27 |
| pTD-*gltA* | *gltA* cloned in pTD-NStrepHis, $Sm^r$ | this study |



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**Table 2. Primer Sequences**

| | sequence (5′ ->3′) |
|---|---|
| Plasmid Primers | |
| *gltA*-FP-pTD | CTGGGAATTCTAATAGGAGGCCACATGGCTGACA |
| *gltA*-RP-pTD | CGCGGATCCTTACTTGCGGTCTTTCAGGGCAACGAT |
| pTD_FP | TGTGTGGAATTGTGAGCGG |
| pTD_RP | ACTTTGTTTTAGGGCGACTG |
| RT-PCR Primers | |
| *gltA*-F | AACCTGCTACCTGCTGCTCAAT |
| *gltA*-R | GCCGACTACACCGCACATCA |
| *aceA*-F | GCGAAGGCTTCTACAAGGT |
| *aceA*-R | GAAGGAAGGCGAGCAGTT |
| *glcB*-F | CGGTCGTTCGTTGCTGTTC |
| *glcB*-R | GTATTCTTGCGGCTGGTGTTG |
| *Icd*-F | GCCGTGCGTGATTATGTGGTA |
| *Icd*-R | GCAGGCATACATACAGGTCCAG |
| *mdh*-F | GCTGTGGATTCAGGTTCCGATA |
| *mdh*-R | TGTCGAGGATTGGCAGGTTGGT |
| *rpoD*-F | GGAACAGGTGGAAGACATCATC |
| *rpoD*-R | TTCAACTGCCGCCAATGC |

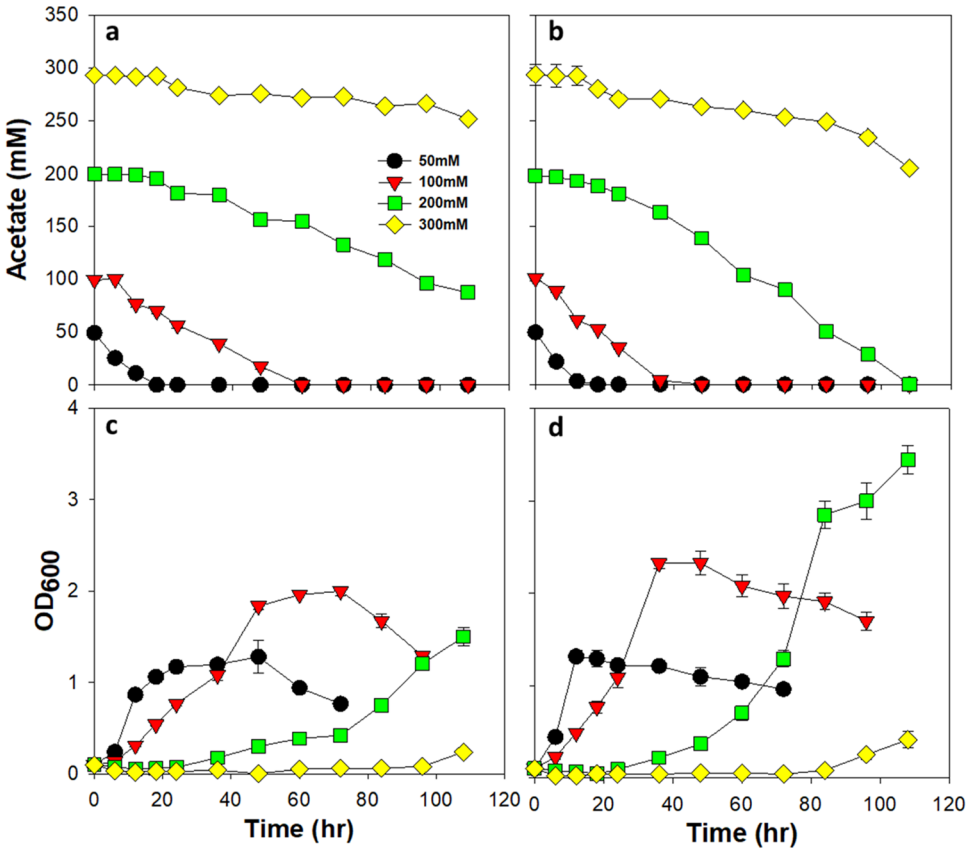


**Figure 2.** *P. putida* KT2440 (WT) growth profiles at different acetate concentrations (50, 100, 200, and 300 mM) and different agitation speeds (100 and 200 rpm). (a) Acetate consumption at 100 rpm, (b) acetate consumption at 200 rpm, (c) cell density at 100 rpm, and (d) cell density at 200 rpm.

(H-ESI) source and a Waters ACQUITY BEH C18 column, was operated at 25 °C with an isocratic flow rate of 0.3 mL $min^{-1}$ of 0.1% formic acid in 3% acetonitrile. Gas samples were taken with a 250 μL syringe and analyzed by gas chromatography (6500GC Agilent Technologies, Young Lin Instrument Co. Anyang, Korea) using a molecular sieve/porapak N column at an oven temperature of 48 °C. Detection was achieved using a flame ionization detector (FID) and a thermal conductivity detector (TCD). The injector, FID, and TCD temperatures were 150, 250, and 100 °C, respectively. The flow rate of the carrier gas (Ar) was 14 mL/min.

For the extraction of PHB (poly-hydroxybutyrate), bacterial cells were harvested by centrifugation at 10,000 rpm for 10 min, washed with distilled water, and the procedure described previously was followed.[28] All experiments were carried out in triplicate to allow statistical data analysis. The average values



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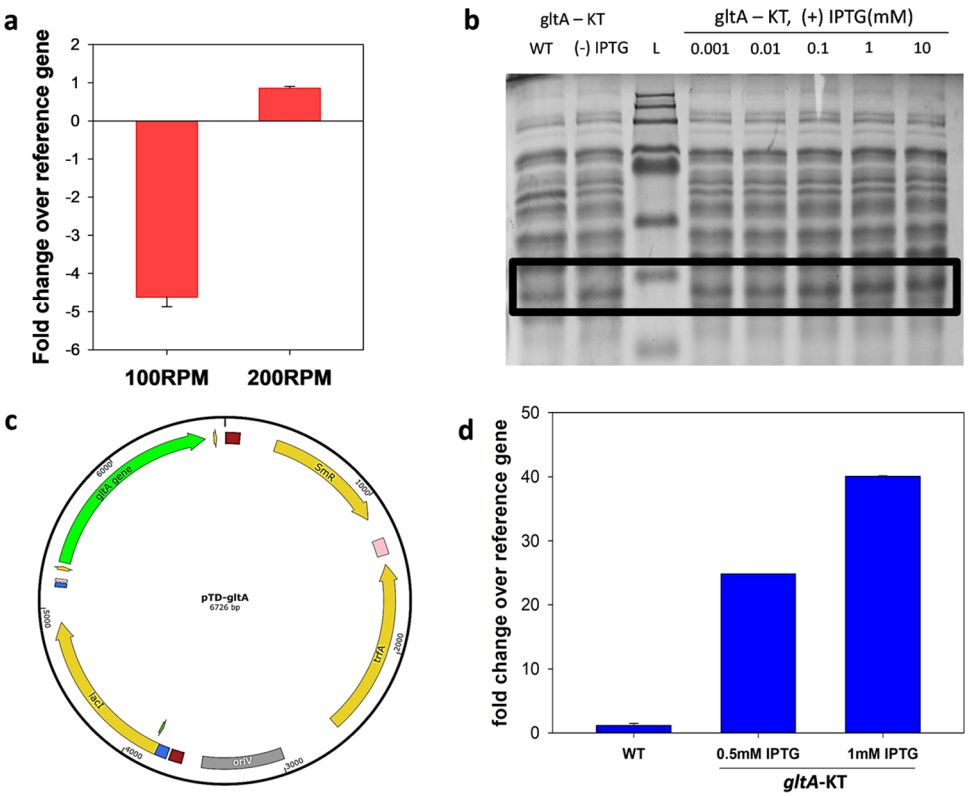


**Figure 3.** Citrate synthase (*gltA*) gene overexpression plasmid construction. (a) *gltA* gene expression levels at different agitation speeds (200 and 100 rpm) quantified by RT-PCR. (b) SDS-PAGE result of citrate synthase gene overexpression at various IPTG concentrations (0.001, 0.01, 0.1, 1, and 10 mM). (c) Plasmid map of *gltA* cloned into the pTD plasmid. (d) Quantification of *gltA* gene expression upon IPTG induction via RT-PCR.

are presented in the graphs, and error bars indicate the standard deviations of the experimentally measured values.

## 3. RESULTS

**3.1. Effect of Agitation Speed on Acetate Uptake and Cell Growth of *P. putida*.** Although acetate is a major metabolite in most bioprocesses, high acetate concentrations are usually toxic to most bacteria and frequently inhibit cell growth.[29] In contrast to more reduced substrates, acetate generally burdens the cellular metabolism, while it requires ATP to assimilate. A relatively oxidized substrate, such as acetate, needs an aerobic environment to take up and generate ATP. The effects of different agitation speeds (100 and 200 rpm) on acetate consumption and cell growth were studied to determine if aerobic respiration alleviates such acetate-induced stress (Figure 2). Both microaerobic (100 rpm) and aerobic (200 rpm) cultivation regimes were compared on different initial acetate concentrations (50−300 mM).

Under aerobic cultivation, the acetate consumption rates were relatively higher than in microaerobic conditions. At 300 mM of initial acetate, ∼88.5 ± 7.1 mM of acetate was consumed under aerobic conditions, whereas it was only ∼41.5 ± 2.4 mM in its microaerobic counterpart during 110 h of cultivation. Lower initial acetate concentrations (50−200 mM) increased the acetate consumption rate in aerobic and microaerobic conditions. In aerobic and microaerobic regimes, 50 mM acetate was consumed at 12 and 18 h, 100 mM acetate at 36 and 48 h, and 200 mM acetate at 96 h and over 108 h, respectively (Figure 2a,b). Acetate consumption and cell growth were significantly lower under 50 rpm or over 300 rpm cultivation (data not shown).

The growth rate is the most important physiological parameter describing the bacterial response to a particular carbon source. In this study, an increase in acetate concentrations from 50 to 100 mM did not affect biomass production. A further increase in acetate concentration to 200 mM retarded growth by ∼25 and 50% under aerobic and microaerobic conditions, respectively, compared to the cells exposed to 50 mM acetate (Figure 2c,d). *P. putida* also exhibited a longer lag phase (24 and 84 h at 200 and 300 mM acetate, respectively). The prolonged lag phase can be attributed to the acetate uncoupling theory,[15,29] in which the higher concentration of acetate anions disrupts the ionic balance within the cell.

Although the acetate consumption and cell growth were higher in aerobic cultivation, the succinate production was higher under a microaerobic environment (1.24 ± 0.17 mM of succinate from 100 mM acetate, 1.74 ± 0.13 mM from 200 mM) compared to an aerobic environment (0.29 ± 0.03 mM from 100 mM, no detection at 200 mM) (Figure 4a). Insufficient oxygen availability in microaerobic conditions appears to cause stringent bacterial metabolism, thereby diverting the pathway toward succinate production. Interestingly, under microaerobic conditions, high initial concentrations of acetate (100 and 200 mM) produced similar amounts of succinate, despite the different times to achieve maximum succinate (36 h for 100 mM vs 96 h for 200 mM). Despite the ability of *P. putida* to metabolize up to 200 mM acetate, maximum succinate production was achieved in the microaerobic culture with 100 mM acetate.

**3.2. Citrate Synthase Gene Expression Characteristics under Microaerobic Conditions.** As reported in *E. coli*,


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microaerobic cultivation may alter gene expressions of metabolic pathways, particularly the TCA cycle and following energy metabolism, via oxygen sensing global regulators (arcA and FNR).[30] Citrate synthase (*gltA*), the first enzyme of the TCA cycle responsible for converting acetyl-CoA into citrate, is believed to be a bottleneck enzyme for acetate uptake (Figure 1). Insufficient expression of this gene may restrict the entry of acetyl-CoA into the TCA/glyoxylate node, influencing the assimilation rate and tolerance to acetate. Therefore, it is essential to determine if the expression of *gltA* of *P. putida* is downregulated during microaerobic cultivation.

The expression levels of *gltA* in aerobic and microaerobic systems (200 and 100 rpm, respectively) were compared. Under microaerobic conditions, the transcript level was ∼5.4 times lower than that under aerobic conditions (Figure 3a). This reduction in *gltA* gene expression in *P. putida* is comparable to that observed in *E. coli* under similar microaerobic conditions.[30] These results suggest that *gltA* expression might be a rate-limiting step in acetate assimilation, leading to a decrease in acetate flux to the TCA cycle in microaerobic environments.

On the other hand, an IPTG-inducible *P. putida* strain (*gltA*-KT) overexpressing *gltA* homologously was developed to investigate succinate production, as described in Section 2.2. SDS-PAGE and RT-PCR analyses showed that IPTG induction increased gltA protein synthesis and transcription significantly (20- and 40-fold increases with 0.5 and 1 mM IPTG, respectively) (Figure 3b,d). Therefore, the developed *gltA*-KT strain was used for subsequent experiments.

**3.3. Succinate Production with Citrate Synthase Overexpression.** Succinate production of *gltA*-KT was compared with that of the wild-type strain using acetate as a sole carbon source, specifically in minimal media devoid of complex components, such as peptone, sorbitol, and yeast extract. The aerobic culture (at 200 rpm) of *gltA*-KT produced 0.73 ± 0.04 mM succinate with 100 mM acetate and 0.78 ± 0.05 mM with 200 mM acetate, whereas the wild-type strain produced lower titers (0.29 ± 0.03 mM succinate from 100 mM, no detection from 200 mM acetate). In microaerobic cultivation (at 100 rpm), *gltA*-KT produced a maximum of 2.64 ± 0.1 mM succinate and 2.26 ± 0.13 mM succinate with 100 mM and 200 mM acetate, respectively (Figure 4a). The *gltA*-KT titers were 2.1 and 1.3 times higher under microaerobic conditions than the wild-type strain (1.24 ± 0.17 mM succinate from 100 mM acetate and 1.74 ± 0.13 mM from 200 mM). The estimated succinate yield of *gltA*-KT was 0.054 ± 0.003 mol/mol at 100 mM acetate, which was almost double that of 200 mM acetate (0.025 ± 0.002 mol/mol) under microaerobic conditions.

*P. putida* is an ideal host for poly-hydroxybutyrate (PHB) production from glucose, fatty acids, and glycerol.[21,31,32] The PHB synthesis pathways are generally related to a common precursor: acetyl-CoA. Recently, *P. putida*, with the overexpression of acetyl-CoA synthetase (*acs*) that converts acetate to acetyl-CoA, increased PHB remarkably.[33] This result suggests that PHB synthesis is a competing pathway of succinate production. The decrease in cellular PHB synthesis was more obvious in *gltA*-KT than in the wild type under aerobic and microaerobic conditions. Specifically, the decrease in PHB under aerobic conditions was significant (12.5% at 100 rpm and 25% at 200 rpm) (Figure 4b). The decrease in PHB synthesis was attributed to the overexpression of *gltA*, which drives the acetyl-CoA flux toward the TCA/glyoxylate cycle.

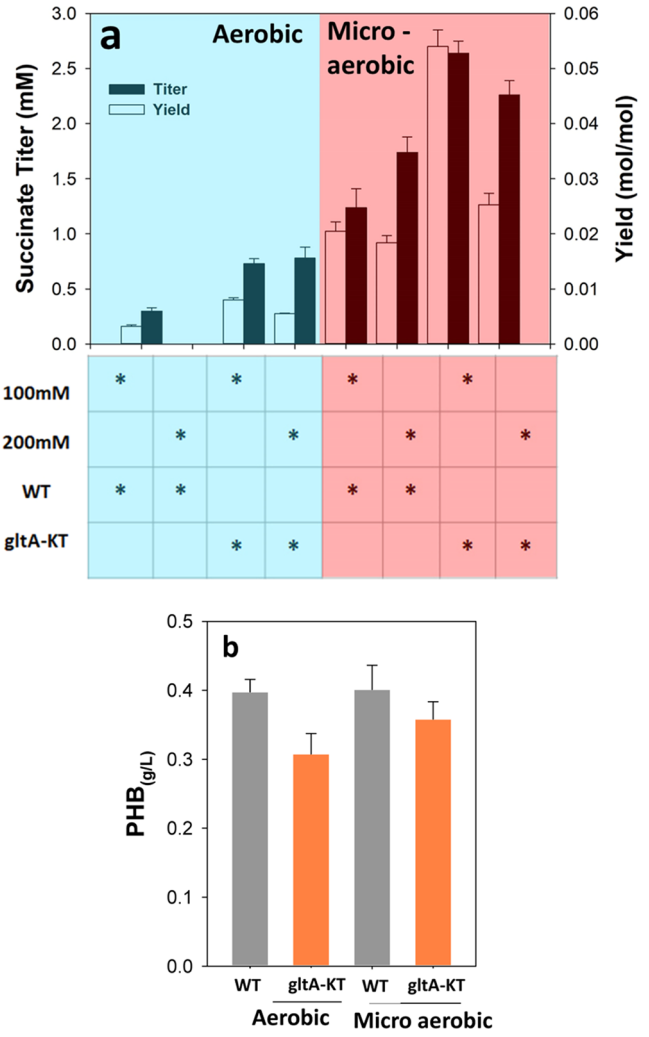


**Figure 4.** Succinate and PHB production from acetate for *P. putida* (WT) and *gltA*-KT strains flask cultivation. (a) Succinate titer and yield at different parameters, such as acetate concentrations (100, 200 mM) and speed (200 and 100 rpm). (b) PHB synthesis of WT and *gltA*-KT using 100 mM acetate at 200 and 100 rpm. *Indicating the presence of operating parameters.

Therefore, *gltA* expression effectively produced succinate by suppressing the PHB pathway, particularly under microaerobic cultivation.

**3.4. Effect of the Overexpression of *gltA* on Glyoxylate and TCA Pathway Enzymes.** The expression of the critical enzymes of glyoxylate and TCA cycles were quantified by RT-PCR: *aceA* (isocitrate lyase), *glcB* (malate synthase), *icd* (isocitrate dehydrogenase), and *mdh* (malate dehydrogenase) (Figure 5a). The *aceA* gene of *gltA*-KT was upregulated to 0.395 (0.5 mM IPTG)- and 1.24 (1 mM IPTG)-fold compared to the wild type. *glcB* was also upregulated to 0.665 (0.5 mM IPTG)- and 0.91 (1 mM IPTG)-fold. The expression levels of the *icd* and *mdh* genes were insignificant.

Metabolite production was analyzed by liquid chromatography−mass spectrometry (LC-MS). α-Keto glutarate significantly decreased in *gltA*-KT (2.53 ± 0.03 mg/L) compared to the wild type (17.69 ± 0.12 mg/L), while malate synthesis increased by *gltA*-KT (4.48 ± 0.02 mg/L vs. 2.46 ± 0.01 mg/L) (Figure 5b). The decrease in α-keto glutarate resulted from *aceA* upregulation in *gltA*-KT because isocitrate is a common


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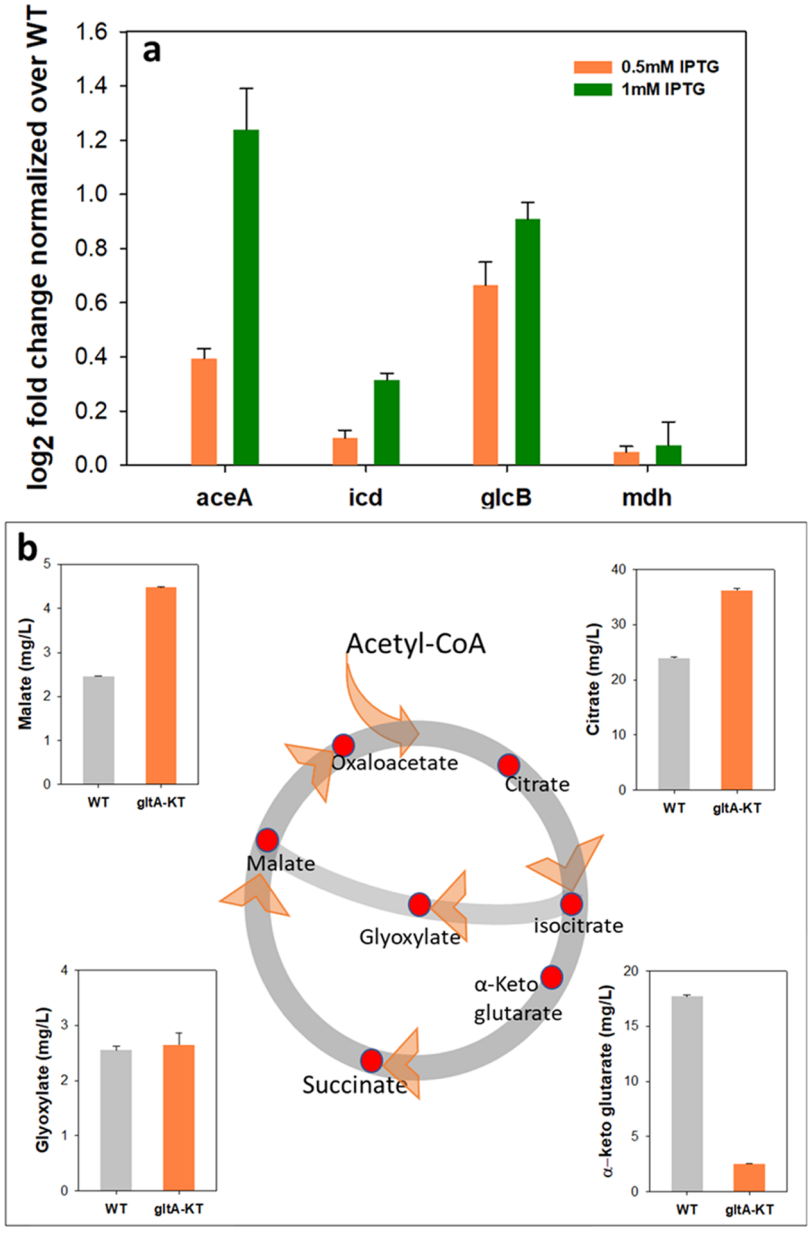


**Figure 5.** Citrate synthase overexpression effect on glyoxylate shunt/TCA cycle genes expression. (a) Relative mRNA expression levels quantification of glyoxylate shunt genes (*aceA*, *glcB*) and TCA cycle genes (*icd*, *mdh*) using RT-PCR. (b) LC-MS data of metabolites released into the medium during acetate metabolism by *P. putida* (WT) and *gltA*-KT.

precursor for isocitrate dehydrogenase (*icd*) and isocitrate lyase (*aceA*) enzymes. The increase in malate and the decrease in the $\alpha$-keto glutarate levels support the upregulation of the glyoxylate pathway with *gltA* overexpression.

**3.5. Optimization of the Culture Conditions and Cofactors for Succinate Production.** The effects of pH and ATP supplementation were examined to improve succinate production by *gltA*-KT. Previous studies reported that the succinate production by yeast and *E. coli* was improved at lower pH, ranging from 5.5 to 6.5, with glucose as the carbon source.[34,35] On the other hand, the free diffusion of acetate ($pK_a$ = 4.75) increased at lower pH because the protonated species ($CH_3COOH$) are higher at lower pH. In contrast, deprotonated acetate ($CH_3COO^-$) requires active transport at the expense of ATP. Therefore, cultivation was carried out at an initial pH of 5.5−7.5. *P. putida* could metabolize acetate above pH 7.0, but consumption did not occur under a pH of 5.5−6.5 (Figure S3b,f). During batch cultivation, the medium pH increased to 8.6 regardless of the initial pH (Figures 6a, S3c,g). The shift in pH to alkaline may limit the acetate-free diffusion and uptake. Therefore, the pH control experiments were carried out with pH 7.0, 7.5, and 8.0. *gltA*-KT at controlled pH 7.5 and 8.0 showed a significant increase in succinate production to 4.73 ± 0.63 mM and 3.35 ± 0.27 mM, respectively, which were ∼4.7 and 3.3 times higher than the wild type (0.99 ± 0.13 mM) and ∼1.3 to 1.8 times higher than *gltA*-KT under uncontrolled pH conditions (Figures 4 and 6b). The results suggest that pH control is essential, and pH 7.5 is the most favorable for succinate production from acetate using *gltA*-KT.

Two ATP equivalents are required to convert acetate to acetyl-CoA.[36] Figure 6c shows the effect of ATP on succinate


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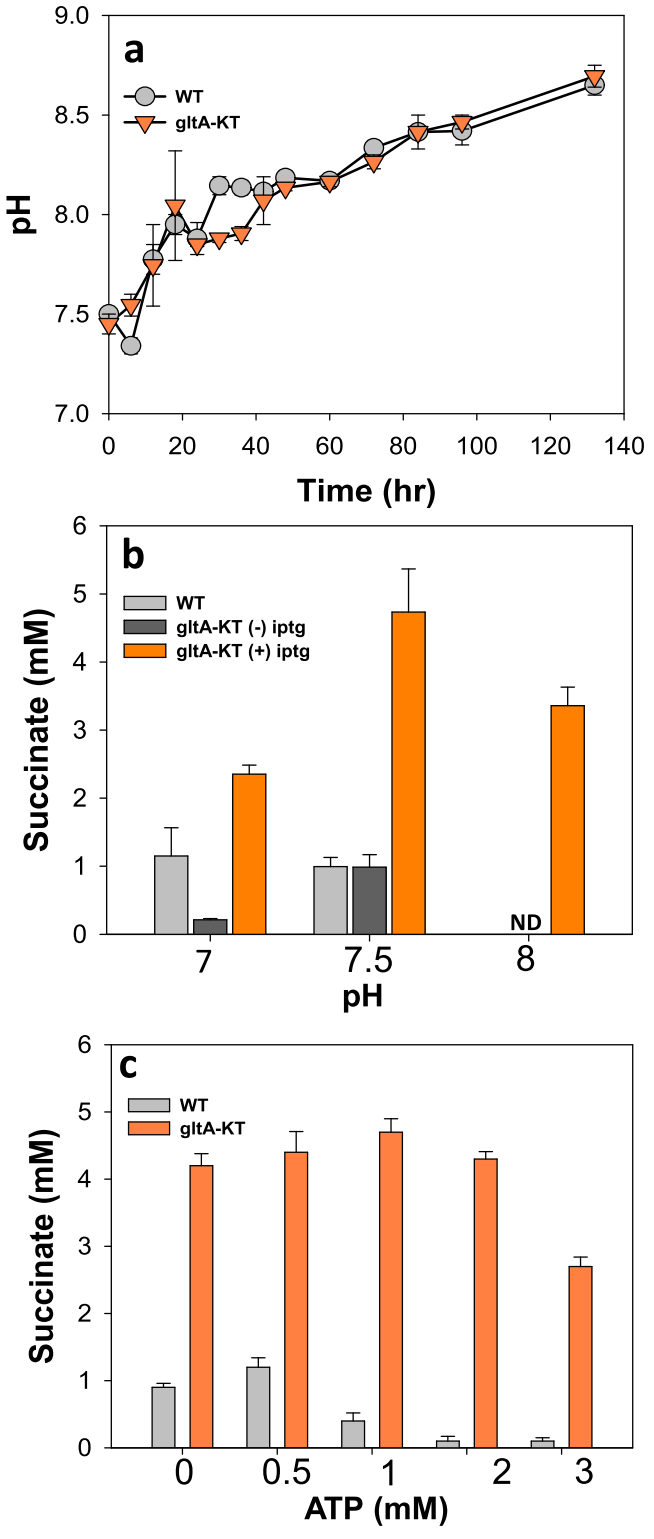


**Figure 6.** Effect of pH on succinate production. (a) Change in pH of the medium during cultivation on acetate. (b) Succinate production at controlled pH (7, 7.5, and 8.0). (c) Maximum accumulated succinates after supplementation of ATP (0.5, 1, 2, and 3 mM) to the medium. ND: Not detected. No succinate production was detected in *gltA*-KT without IPTG addition and the wild type (WT) at pH 8.0.

production. Adding 1 mM ATP produced a marginal enhancement in succinate production (~0.5 mM) of *gltA*-KT at a controlled pH of 7.5 compared to *gltA*-KT without ATP supplementation (Figure 6c). On the other hand, a further increase in ATP (2 and 3 mM) caused a gradual decrease in succinate production in both the wild type and *gltA*-KT.

**3.6. Succinate Production from Resting Cells and Other Carbon Sources.** Carbon balance analysis of growing cells showed that most of the carbon within acetate was distributed to biomass and $CO_2$ (Table 3). The theoretical maximum yield of 0.5 mol succinate/mol acetate might be achieved with nongrowing cells because carbon flux is directed primarily toward product synthesis rather than biomass formation under nitrogen-limiting conditions. Nitrogen is essential for cell growth but not for succinate production. Under such conditions, the succinate biosynthesis increased slightly to 4.94 ± 0.04 mM with an initial acetate concentration of 200 mM, which is similar to the conditions where the pH is controlled 7.5 (4.73 ± 0.63 mM) with *gltA*-KT (Figure 7). The lower titer indicated that approximately 90% of the carbon in acetate is not recovered to succinate on bioconversion. The nitrogen-limited environment may favor producing PHB[32] or other unknown metabolites. Furthermore, adding yeast extract (0.2 g/L) did not alter the succinate production of resting cells.

**Table 3. Estimation of Carbon Recovery of *gltA*-KT**

| substrates and metabolites | carbon balance (mmol) |
|---|---|
| Substrate (consumed) | |
| acetate[a] | 8 |
| Metabolites (produced) | |
| succinate | 0.94 |
| biomass[b] | 3.33 |
| $CO_2$ | 2.6 |
| carbon recovery % | 85.8% |

[a]Consumed acetate was chosen at the time point where maximum succinate accumulated. Carbon balance (mmol) calculations were based on the culture volume of 50 mL. [b]Biomass is estimated with the cell dry weight (mg/L) and $C_5H_7NO_2$ formula.

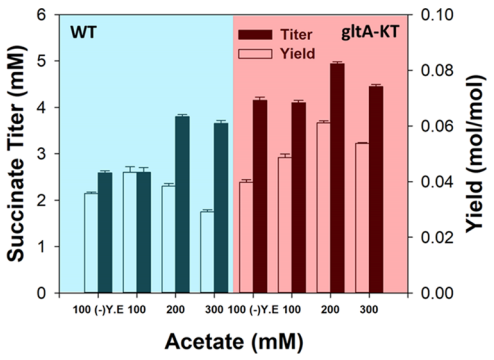


**Figure 7.** Succinate titer and yield by the resting cell system of *P. putida* (WT) and *gltA*-KT using various initial acetate concentrations (100, 200, and 300 mM). One control experiment was conducted without adding yeast extract [(−) Y.E] with 100 mM acetate to understand the importance of yeast extract in succinate production during resting cell acetate transformation. The cell density was approximately 3.0 ($OD_{600}$). The incubation time was altered depending on the acetate consumption rate. (−) Y.E indicates the absence of yeast extract.

These results suggest that *P. putida* might not improve succinate conversion in a nitrogen-limited environment using acetate as a feedstock, unlike *E. coli*.[5] Succinate production was


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compared between glucose and acetate, where glucose is a favorable carbon source for succinate production and energy (ATP) metabolism (Figure 8).[37] The succinate titer with acetate (4.73 ± 0.63 mM) was approximately 50% higher than that of glucose (2.3 ± 0.05 mM).

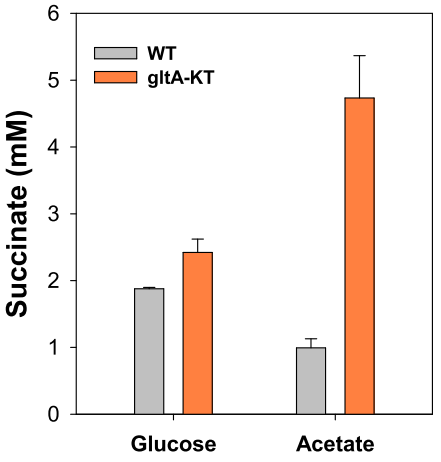


**Figure 8.** Comparison of succinate production with glucose (40 mM) and acetate (100 mM) as the carbon source at 100 rpm (microaerobic).

## 4. DISCUSSION

Currently, the feedstock of biosuccinate production is limited to glucose. Renewable carbon sources, such as glycerol and acetate, have been highlighted to produce succinate. Acetate might be an alternative and preferable carbon source for *P. putida* over glycerol and glucose because of its abundance and cost-effectiveness.[38] The natural tolerance of *P. putida* (without genetic engineering or adaptive evolution) to acetate is greater than *E. coli*; *P. putida* is tolerant to 200 mM, whereas *E. coli* is only 60 mM (5 g/L).[39] All experiments in this study were carried out in a minimal medium with a low amount of yeast extract (0.2 g/L). One of the unique aspects of this study is the use of acetate as the sole carbon and energy source, unlike previous experiments with *E. coli* that typically employed a rich medium like SMAC (peptone—20 g/L, sorbitol—10 g/L) and/or yeast extract (2 g/L). Consequently, the observed lower succinate titers and the maximum theoretical succinate yield (~9.5%) can be attributed to the fact that bacterial biomass formation and succinate production were solely dependent on acetate. The primary focus of our study was to investigate whether *P. putida* is capable of consuming higher concentrations of acetate (100−200 mM) under limited oxygen conditions. Additionally, we aimed to determine the potential for succinate production of *P. putida*. Specifically, this study investigated the role of citrate synthase under microaerobic conditions and provides preliminary evidence that *P. putida* can produce succinate and consume higher acetate concentrations under microaerobic conditions. This research holds significance in shedding light on the metabolic capabilities of *P. putida* under oxygen-limited conditions.

The acetate consumption pattern of wild-type *P. putida* is of interest to further develop a recombination strategy. Previous studies have shown that *P. putida* consumes acetate using a carbon electrode as a terminal electron acceptor, even under anaerobic conditions.[40] In the present study, regardless of the cell generated throughout cultivation, the cell takes up approximately 22 mM acetate every 12 h under microaerobic conditions (100 rpm) for 100 mM acetate (Figure 2). Metabolic pathway engineering should be accompanied by pH control to enhance acetate assimilation. Therefore, *gltA* overexpression can facilitate a higher acetate influx into the TCA cycle in such pH-controlled cultivation (Table 4).

Compared to the wild type, acetate consumption of *gltA*-KT decreased at 100 and 200 rpm. On the other hand, the specific acetate consumption (mM/CDW) of the *gltA*-KT strain was increased slightly, as shown in Figure S1, suggesting that acetate consumption per cell was higher than that in the wild type. The *gltA* overexpression reduced the cell growth of *gltA*-KT by 20% compared to wild-type *P. putida*. The lower cell density of *gltA*-KT resulted in lower acetate consumption (Figure S2). In addition, *gltA* overexpression did not promote growth at higher acetate concentrations (300 mM) (data not shown). Further optimization and process control strategies, as well as strain development, will be necessary to improve the acetate assimilation rate.

Unlike *E. coli*, acetate conversion to succinate was observed using wild-type *P. putida* in minimal media in this study.[5] There is a possibility that the cell switches to "overflow metabolism," particularly when exposed to higher concentrations of acetate (100 and 200 mM) under microaerobic conditions. An overflow metabolism often occurs because of an imbalance between a high substrate uptake rate and limited catalytic activity in the TCA cycle. The LC-MS results for metabolite analysis support the overflow metabolism (Figure 5b) in *P. putida*.

**Table 4. Comparison of the Succinate Titers from Acetate with Other Strains**

| bacteria | titer (mM) | operation mode | growth medium | refs |
|---|---|---|---|---|
| *E. coli* MG1655 (Δ*sdhAB*,Δ*iclR*,Δ*maeB*, Δ*poxB*, *gltA*) | 16 | aerobic | 5 g/L sodium acetate, 2 g/L yeast extract, SMAC medium, 220 rpm (**rich medium**) | 5 |
| *E. coli* MG1655 (Δ*iclR*Δ*sdhAB*Δ*maeB acs- gltA-acnB*) | 11.23 | aerobic | 50 mM sodium acetate, 2 g/L yeast extract, SMAC medium, 220 rpm (**rich medium**) | 41 |
| *E. coli* MG1655 (Δ*iclR*Δ*sdhAB*Δ*maeB gltA-fdh*) | 30.9 | aerobic | 5 g/L sodium acetate, 2 g/L yeast extract, SMAC medium, 220 rpm (**rich medium**) | 42 |
| *P. putida* KT2440 | | | | |
| WT | 1.24 ± 0.17 | microaerobic | 100 mM sodium acetate, 0.2 g/L yeast extract, 100 rpm (**minimal medium**) | this study |
| *gltA*-KT | 2.64 ± 0.11 | | | this study |
| *gltA*-KT (pH 7.5) | 4.73 ± 0.63 | | | this study |



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Achieving higher succinate production will require control of the succinate dehydrogenase (*sdhAB*) activity because the cells can reassimilate succinate using the *sdhAB* enzyme. Thus, deletion or downregulation of *sdhAB* might improve apparent succinate production. Microaerobic cultivation could reduce *sdhA* enzyme activity to some extent.[43] Figure S6 provides valuable insights by showing that the expression of the *sdhA* gene in *P. putida* wild-type was reduced by approximately 2.2-fold during microaerobic cultivation compared to aerobic conditions. This reduction in the gene expression levels of *sdhA* highlights the importance of controlling the *sdhA* genes to enhance succinate yields. Under oxygen-limited conditions, global regulators sense the cellular redox state and negatively regulate the gene transcription of *sdhAB* involved in succinate conversion.[30] Succinate synthesis can be optimized, and higher yields can be achieved by controlling the activity and expression levels of *sdhAB*. Understanding the regulatory processes involved in succinate metabolism is crucial to enhance succinate production in *P. putida*.

The pH range of 7.0−8.0 used in this study favored PHB synthesis. The related enzymes of *Pseudomonas* sp. performed better at neutral pH (7−7.5) in a previous study.[44] Figures 4 and S6 suggest that PHB synthesis was dominant under microaerobic cultivation as only 12.5% PHB was reduced, and only one fold reduction in PHB synthase (*phaC*1 and *phaC*2) was observed. The majority of the carbon (85.5%) was recovered to the biomass and $CO_2$ (Table 3); hence, fine-tuning the biosynthesis and succinate production phase may improve the succinate yield further.

Developing bioprocesses to produce platform chemicals depends on the operating costs and the price of target products. This study suggests that the overexpression of *gltA* in *P. putida* KT2440 produces higher succinate titers under microaerobic cultivation, alleviating the major concern of energy-intensive aeration (Figures 2a,b and 4). Using minimal media is another advantage in further reducing operating costs. This engineered *P. putida* strain can produce higher yields of succinate from acetate using a further metabolic engineering strategy. Furthermore, fine-tuning and engineering the expression of PHB synthase, succinate dehydrogenase, and succinate exporter can optimize the strain to achieve the maximum theoretical yields and an economically viable succinate production process.

## 5. CONCLUSIONS

This study assessed the potential for acetate as an alternative feedstock for succinate production by *P. putida* under microaerobic conditions. Citrate synthase (*gltA*), one of the bottleneck enzymes to metabolize acetate into the TCA cycle, was overexpressed in *P. putida*. The constructed recombinant strain *gltA*-KT produced ∼4.6 ± 0.6 mM succinate at a pH of 7.5, achieving ∼9.5% of the theoretical maximum succinate yield compared to the wild type (1.24 ± 0.17 mM and ∼2.5%). The *gltA* overexpression resulted in ∼400% higher succinate production than the wild type. The engineered strain of *P. putida* has great potential for producing succinate from acetate compared to glucose (2.3 ± 0.05 mM). The microaerobic cultivation of *P. putida* could contribute to developing a techno-economical process of succinate production from renewable carbon sources, such as acetate.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c02520.

Figure S1. specific acetate consumption of the wild type and *gltA*-KT; Figure S2. total acetate consumption and cell densities of *P. putida* (WT) and *gltA*-KT; Figure S3. effect of initial pH on acetate consumption and succinate production of *P. putida*; Figure S4. growth of the resting cell of the wild type and *gltA*-KT.; Figure S5. $CO_2$ gas profile; Figure S6. expression of *sdhA*, *phaC*1, and *phaC*2 transcripts in the wild-type strain; and Figure S7. viability of cells over multiple generations (PDF)

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### Notes

The authors declare no competing financial interest.

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