Alcohol Dehydrogenase (adhE) Metabolic Gene Knockout in *Escherichia coli* GEM Increases Succinate Production from Glycerol

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**Abstract**

Genome-scale metabolic model (GEM) of *Escherichia coli* has been published with applications in systems metabolic engineering for strain development on different carbon sources and directing biological discovery. The use of glycerol as an alternative carbon source is economically viable in bio-refinery. The use of GEM for predicting metabolic gene deletion of alcohol dehydrogenase (adhE) for increasing succinate production in *E. coli* from glycerol substrate remained largely unspecified. Here we hypothesized that metabolic gene knockout of adhE in *E. coli* from glycerol could increase succinate production. We constructed a proof-of-principle strain BMS6 (ΔadhE), by predicting increased succinate production in *E. coli* GEM and confirmed the predicted outcome using wet cell experimentation. The mutant GEM (ΔadhE) predicted 11% increase in succinic acid production from glycerol compared to its wild-type model (iAF1260) and the *E. coli* BMS6 (ΔadhE) produced 1.05 g/L and its corresponding wild-type produced 0.04 g/L (nearly 23 fold increase). The *E. coli* BMS6 (ΔadhE), confirmed the above hypothesis, and further demonstrate that *E. coli* GEM can prospectively and effectively predict new metabolic gene deletion target from glycerol substrate and could ultimately serve as platform for new strain design strategies and biological discoveries.

**Keywords:** Succinic Acid Production; *Escherichia coli* Genome-Scale Metabolic Model; Proof-of-Principle Strain; Metabolic Gene Knockout; Alcohol Dehydrogenase (adhE); Glycerol

**Introduction**

The *Escherichia coli* genome-scale metabolic models (GEMs) has been published [1,2] and their respective applications in strain design and biological discovery have been extensively reported in the literature [3-6]. The ability of GEMs to accurately predict metabolic gene knockout that could tally with wet cell experimentation on different carbon sources remained a challenge, as it depends on the scope and quality of the GEM used in simulation. Production of platform chemicals such succinic acid via bio-based routes would require construction of metabolic engineering strains from different carbon sources, such as glycerol, which is cheaper and considered economically viable in bio-refinery process [7]. In any strain design strategy, construction of proof-of-principle strain at the beginning using alternative carbon sources is of utmost importance, more especially with the matured field of genome-scale science gaining attention and facilitating new discoveries in recent years [5,8-11].

*Escherichia coli* GEM has been used for identification of novel metabolic gene(s) knockout(s) targets for increasing succinic acid production from glucose, and glycerol carbon sources [12-17]. The genes targeted for knockouts include atpE encoding F$_{0}$ membrane bound

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ATP synthase [16], fdoH encoding formate dehydrogenase-O [17], and gnd encoding 6-phosphogluconate dehydrogenase [15]. Glucose and glycerol carbon sources were utilized as substrates for construction of three (3) proof-of-principle strains of E. coli using GEM for increasing succinate production. These strains were designated as BMS1 (ΔatpE) BMS2 (Δgnd) and BMS4 (ΔfdoH) as previously reported in their original documentations [15-17]. Other researchers reported the deletion of alcohol dehydrogenase (adhE) and other target genes for increasing succinic acid production in E. coli using glucose as carbons source [18]. In addition, direct production of 1,4-butanediol (BDO) in E. coli reported deletion of alcohol dehydrogenase, because it catalyzes a competing reaction for NADH regeneration [19]. In a different study reported elsewhere, adhE was deleted to preserve NADH and channeled fluxes towards 1-butanol production in E. coli from glucose substrate [20,21]. We reasoned that the metabolic gene knockout of alcohol dehydrogenase (adhE) could increase succinic acid production under anaerobic condition in E. coli, because it is capable of generating additional NADH required for succinic acid formation [22]. Under normal circumstances, 2 moles of NADH is required to form 1 mole of succinic acid from glucose substrate under anaerobic condition in E. coli [22]. It is currently unclear whether the deletion of adhE that consumes NADH in E. coli anaerobic metabolism of glucose, can increase succinic acid production when glycerol is used as an alternative carbon source in lieu of glucose.

Here we report the use of E. coli GEM [1] for predicting increase in succinic acid production by knocking out adhE from glycerol substrate. We hypothesized that knocking out adhE in E. coli under anaerobic conditions from glycerol substrate could increase succinic production. Furthermore, we experimentally confirmed the predicted outcome using wet cell experiment and constructed a proof-of-principle strain designated as E. coli BMS6 (ΔadhE). The proof-of-principle strain constructed in this study and elsewhere [15-17] could guide future strain design strategies for succinic acid production when glycerol is selected as substrate in the context of bio-refinery.

Materials and Methods

In silico analysis of gene knockout

Escherichia coli genome scale stoichiometric model iAF1260 [1] was employed for the in silico simulation of gene deletion by using Minimization of Metabolic Adjustment (MOMA) algorithm [23] with OptFlux software platform (http://www.optflux.org) [24]. The E. coli iAF1260 model has been tested and proven to be predictive for computations of growth rates and metabolite excretion rates from a range of substrates and genetic conditions [1,9]. MOMA was described as a flux based analysis technique that employs quadratic programming to search for the nearest point in the feasible solution space of the mutant model in relations to its wild-type optimal point feasible solution space [23]. The OptFlux software platform is an in silico metabolic engineering (ME) platform that was implemented using the Java programming, which contains MOMA as a simulation algorithm. Flux balance analysis (FBA) was used for all phenotype simulations. All the simulation of the mutant and the wild-type models were performed using the OptFlux software version 3.07.

Glycerol was used as solitary carbon source under anaerobic conditions. The substrate uptake rates was constrained to a maximum of 18.5 mmol gDW⁻¹ h⁻¹ whereas its corresponding oxygen uptake rates was set to zero, as the environmental condition was anaerobic. These values were selected based on closely established experimental observations on aerobic and anaerobic growth in E. coli [25,26].

Bacteria and Plasmid

E. coli JM109 (F⁻ traD36, proAB+ lacI², D (lacZ) M15) endA1 recA1 hsdR17 (rK⁺, mK⁻) mcrA supE44 l- gyrA96 relA1 D (lacproAB) thi-1) was used for maintenance of the pKD4 and pKD46 plasmids. The plasmids were used strictly following the method described previously [27]. The plasmid pKD4 was extracted from E. coli JM109 using the QIAprep Miniprep kit according to the manufacturer’s protocols.

Media chemicals and other reagents

E. coli cells used in this study were grown in LB medium containing 0.5% yeast extract (Difco), 0.5% NaCl and 1% Bacto tryptone (Difco) without or with antibiotics at the concentrations of 100 µg/ml ampicillin and 30 µg/ml of Kanamycin. L-arabinose, and glycerol were obtained from SigmaAldrich. KAPA HiFi Hotstart Ready Mix (2X) was from KAPA BIOSYSTEMS. Agarose was purchased from (Sigma Aldrich).

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**PCR Primers**

The *E. coli* adhE gene sequence was used to design forward and reverse primers with pKD4 template plasmid sequence. The primers had 45-nt 5' extension including the gene initiation codon (H1) and 20-nt sequence (p1) as described previously [27,28]. Table 1 gives the details of the primers used in this study.

<table>
<thead>
<tr>
<th>E. coli strains</th>
<th>Relevant characteristics or genotype</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>JM109</td>
<td>Wild-type (F¢ (traD36, proAB+ lacIq, D (lacZ) M15) endA1 recA1 hsdR17 (rk_, mk+) mcrA supE44 → gyrA96 relA1 D (lacproAB) thi-1)</td>
<td>Lab collection</td>
</tr>
<tr>
<td>BMS5</td>
<td>ΔldhA: FRT-Kan-FRT</td>
<td>This work</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pKD4</td>
<td>Bla FRT-kan-FRT (template plasmid for FRT-flanked kanamycin resistant gene; Amp&lt;sup&gt;8&lt;/sup&gt;, Km&lt;sup&gt;9&lt;/sup&gt;)</td>
<td>(Datsenko and Wanner, 2000) [27]</td>
</tr>
<tr>
<td>pKD46</td>
<td>Bla γ β exo (red recombinase helper plasmid), temperature-conditional replicon (Red recombinase expression vector; Amp&lt;sup&gt;8&lt;/sup&gt;)</td>
<td>(Datsenko and Wanner, 2000) [27]</td>
</tr>
<tr>
<td>Primer set</td>
<td>Sequences 5' – 3'</td>
<td></td>
</tr>
<tr>
<td>adhE_F</td>
<td>ATGGCTGTACTAATGCCTGACTAAAGGACTGCCGTGTAGGAGCTGCTTC</td>
<td>This work</td>
</tr>
<tr>
<td>adhE_R</td>
<td>TTAAGCGGATTTTTTTTCGCTTTTTTCTCAGCTTTTAGCGGGAGGCAGC CATATGACTATCCCTCCTTAG</td>
<td>This work</td>
</tr>
</tbody>
</table>

**Table 1**: *Escherichia coli* strains, plasmids, and primers used in this work.

**Generation of PCR fragments**

PCR reactions were carried out in an Eppendorf thermo cycle using 25 µl reactions containing 12.5 µl of KAPA HiFi Hotstart Ready Mix (2X), 1 µl of pKD4 template DNA, 1.0 µl of each primer. Reactions were performed for 30 cycles: 95°C for 3 min, 98°C for 20 sec, 55°C for 15 sec, 72°C for 1:30 sec, 72°C for 60 sec and cooling at 4°C. PCR products were purified using SV gel and PCR clean up system (Promega, USA), according to the manufacturer's protocol. Then, the PCR products obtained were analyzed by 1% agarose gel-electrophoresis using 1X Tris-acetate buffer.

**Electroporation and mutant selection**

*E. coli* JM109 harboring the λ-Red helper plasmid pKD46 was grown in 100 ml of LB medium with ampicillin and 1 mM L-Arabinose at 30°C to an OD<sub>600</sub> of 0.3. Competent cells for electroporation were prepared as described previously [29]. A 1.0 µl (400 ng) aliquot of the PCR fragment was mixed with 50 µl of competent cell in an ice-cold Eppendorf electroporation cuvette (0.2 cm). Electroporation was performed at 2.5 KV with 2 mF and 600Ω and was followed by immediate addition of 1 ml of SOC medium (0.5% yeast extract (Difco), 2% Bacto tryptone (Difco), 2.5 mM KCl, 10 mM NaCl, 10 mM MgCl<sub>2</sub>, 10 mM MgSO<sub>4</sub> and 20 mM glucose) with 1 mM L-arabinose. The SOC medium mixed with the electroporated cells was incubated for 2 hours at 37°C. Selection of kan<sup>R</sup> transformant was followed immediately by spreading one-tenth portion of the electroporated cells onto kanamycin agar plates as described by Baba and colleagues [28]. To test for accurate mutational inactivation or correct chromosomal structure, 20 µl PCR verification method was conducted with kanamycin specific primers K1 and K2 as described earlier [27].

**Anaerobic fermentation**

Bacterial cells starter culture was made by growing the cells in 10 ml LB medium with shaking at 200 rpm at a temperature of 37°C. One milliliter of seed culture was used to inoculate a 125 ml butyl rubber stoppered serum vial, which contained 100 ml of fermentation broth.

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media as described by Lee and colleagues [30] with slight modification, replacing glucose with glycerol (10 g l⁻¹). The fermentation media used contained the following ingredients (per liter): yeast extract = 5 g; glycerol = 10 g; NaHCO₃ = 10 g; NaH₂PO₄.H₂O = 8.5 g; K₂HPO₄ = 15.5 g (pH = 7.0). Anaerobic conditioning was established by filling the headspace with N₂ and addition of Na₂S.9H₂O (final 1 mM). Cells were cultivated under anaerobic conditions at 37ºC with shaking at 200 rpm for 6 days unless otherwise stated.

**Analytical procedure**

The concentrations of glycerol, ethanol and organic acids (lactate, formate, and succinate) were quantified by high performance liquid chromatography using the Agilent 1260 Infinity (Agilent Technologies, USA). The HPLC Agilent, equipped with an RI detector and a 300 x 7.88 mm Aminex HPX-87H ion-exchange column (Bio-Rad laboratories, USA), was used for these purposes. The culture supernatant was passed through a syringe filter (pore size of 0.2 µm) after centrifugation at 10,000 x g for 10 min and stored at -20ºC for analyses. To optimize peak separation with glycerol carbon source, the column was eluted isocratically at 42ºC with a flow rate of 0.6 ml min⁻¹ using 30 mM H₂SO₄ as the mobile phase in accordance with methods previously described [31,32]. To quantify cell growth, the optical density of the cell cultures was measured at 600 nm using a GENESYS 105 VIS spectrophotometer (Thermo scientific, USA).

**Results and Discussion**

Mixed acid fermentation reaction in *E. coli* under anaerobic conditions, produces ethanol, succinate, acetate and formate using different substrates [33]. As a platform chemical with varieties of applications, succinic acid can be produced via bio-based routes from glycerol by metabolically engineered *E. coli* strain. This form of succinic acid production is often regarded as a green technology in the context of bio-refinery. GEMs of *E. coli* is recently considered foundational in strain design strategies [34] and biological discovery [4,10]. Construction of proof-of-principle strain (taking into account economically cheaper substrate, like glycerol) is a fundamental requirement for industrial strain design strategies [34]. The knocking out of adhE in *E. coli* on glucose substrate was established to increase the pool of NADH availability, which is a precursor for succinic acid production under anaerobic conditions [18,23]. On the bases of the aforementioned fact, we then hypothesize that knocking out of adhE in *E. coli* using glycerol substrate (see Figure 1) could increase succinate production. The current study is intended to confirm the above hypothesis that the removal of the target gene (adhE) could increase succinate production in *E. coli* under anaerobic condition from glycerol substrate (See Figure 1). The current work confirmed that the removal of adhE using GEM, predicted increased succinate production in *E. coli* from glycerol substrate and experimentally verified using wet cells (See Table 2 and 3).

**Figure 1:** Metabolic pathways involved in anaerobic glycerol metabolism in *E. coli* (Mienda et al., 2015b; Zhang, Shannugam, and Ingram, 2010a). The metabolic gene knockout of alcohol dehydrogenase (adhE) is shown in red. The oxidation of NADH to NAD⁺ was blocked by knocking out adhE in strain BMS6 (ΔadhE), thereby preserving additional NADH that is needed for increasing succinate production from glycerol substrate. Relevant reactions involved in glycerol metabolism in *E. coli* for succinate production are indicated by the names of their gene(s) in italics.
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### Table 2: In silico prediction results for increasing succinate production in E. coli GEM.

<table>
<thead>
<tr>
<th>Knockout genes</th>
<th>Biomass (h⁻¹)</th>
<th>% Biomass</th>
<th>Succinate (mmol gDW⁻¹ h⁻¹)</th>
<th>% succinate</th>
<th>Ethanol (mmol gDW⁻¹ h⁻¹)</th>
<th>Acetate (mmol gDW⁻¹ h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT Feist model</td>
<td>0.34070391</td>
<td>100.0</td>
<td>0.11371</td>
<td>100.0</td>
<td>23.77531</td>
<td>5.49531</td>
</tr>
<tr>
<td>adhE/b1241</td>
<td>0.2303747</td>
<td>68.1</td>
<td>0.12627</td>
<td>111.0</td>
<td>23.81368</td>
<td>5.50755</td>
</tr>
</tbody>
</table>

Table 3: Succinate production from glycerol by proof-of-principle strain E. coli BMS6 (ΔadhE) and its wild-type during anaerobic vial fermentation.

- Data represent the averages of three samples (mean ± standard deviations) taken from days of anaerobic fermentation cultures supplemented with 10 g/l of glycerol.
- Anaerobic vial fermentation on 10 g/l initial glycerol for 6 days.
- Calculated by subtracting the initial glycerol concentration from the residual glycerol concentration.

The in silico prediction result with E. coli GEMs shows up to 11% higher increase in succinic acid production relative to the wild-type model when glycerol substrate is used (See Table 2). The corresponding wet cell experiment shows direct increase in succinic acid production as hypothesized (see Table 3). The experimental outcome of the current work confirmed that knocking out of adhE in E. coli from glycerol substrate under anaerobic condition increases succinic acid production (see Table 2 and 3). To demonstrate the utility of E. coli GEM, we constructed a proof-of-principle strain named E. coli BMS6 (ΔadhE) with overall increase in succinate production that is nearly 23 fold compared to its corresponding wild-type counterpart after 6 days of fermentation with glycerol substrate (see Table 3). Anaerobic glycerol fermentation in E. coli has been previously established [32] to have succinate, and ethanol as the major fermentative end products of glycerol metabolism, and it produces twice the number of reducing equivalent when compared to glucose substrate [32]. This is evident by the high amount of succinate (23 fold increase) and ethanol (10 fold increase) produced in this study (see table 2). This could be the plausible explanation for having 23 fold increase in succinic acid production in BMS6 (ΔadhE) proof-of-principle strain constructed in this work. It has been identified that the major bottleneck in anaerobic succinate production in E. coli is NADH limitation [22], therefore deletion of NADH consuming reaction and/or enzyme that catalyzes such reaction could increase the availability of NADH that would be directed towards succinate production. It has been previously established that E. coli glucose metabolism consumes 2 moles of NADH for every 1 mole succinic acid produced under anaerobic conditions [22]. On the bases of foregoing, we reasoned that since glycerol is more reduced than glucose and can produce twice the number of reducing equivalent when compared with its glucose counterpart, therefore, the use of glycerol as an abundant feedstock could be an interesting substrate to explore.

Furthermore, anaerobic glycerol metabolism in E. coli was reported to produce high amounts of ethanol, and succinic acid, as major fermentative end products, constituting about 93% in terms of molar bases (86% ethanol and 7% succinic acid) and only minor amount of acetate are formed with no detectable formate or lactate [32]. These findings are in conformity with the results obtained in this study.

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(see Table 3) and it conspicuously shows that GEM of *E. coli* iAF1260 used in this study, prospectively and effectively predicted increase succinic acid production from glycerol substrate (see table 2 and 3). Ethanol production decreases by 55% in the proof-of-principle strain constructed BMS6 (Δ*adhE*) when compared to its wild-type counterpart (see table 3), this is because deletion of *adhE* is expected to stop the oxidation of NADH to NAD⁺ (see Figure 1), which is also required for anaerobic ethanol production in *E. coli*. The mechanism involved in production of ethanol in strain BMS6 (Δ*adhE*) is intriguing. Because anaerobic dissimilation pathway for glycerol metabolism in *E. coli* (see Figure 1) differs considerably with that of glucose, production of succinate and ethanol as NADH requiring reactions will vary dramatically when glycerol is used as carbon source. This is largely due to exclusively reduced nature of glycerol synthesizing highly reduced fermentative end product, such as ethanol and succinate [32]. In addition, knocking out of *adhE* in *E. coli* from glucose using wet cell experiment was previously reported in *E. coli* strain XZ15, but the mutant strain produced only succinic acid concentration (5.9 g/L) that was 9% higher than its corresponding wild-type strain (5.4 g/L) [35]. The strain constructed *E. coli* BMS6 (Δ*adhE*) in this study using glycerol substrate produces nearly 23 fold (1.051 g/L) increase relative to its wild-type counterpart (0.045 g/L) (see table 3). This could be attributed to the reduced nature of glycerol and its ability to produce twice the number of reducing equivalents produced by its glucose counterpart [32].

Glycerol metabolism in *E. coli* remained slow with up to six (6) days of fermentation as reported previously [36]. The previous finding is in conformity with the result obtained in this study (see table 3), as slow glycerol metabolism follow different routes compared to glucose, and the slow metabolism and impaired growth rate seen in this study have been attributed to redox imbalance resulting from the use of certain intermediate for biosynthesis, and/or limited energy for cell envelope biosynthesis in a process called gluconeogenesis [16,36]. The strain constructed in this study BMS6 (proof-of-principle strain) is not considered as a final industrial strain in terms of yield, titer, and productivity, but rather considered as a starting point for preliminary identification of novel gene knockout targets when glycerol is used as substrate. The careful selections of chassis strain (e.g. *E. coli*), and cheaper carbon substrates (e.g. glycerol), are the basic fundamental requirements in systems strategies for industrial strain development [34]. It is important to emphasize that *E. coli* GEMs used for different strain design strategies, and proof-of-concepts studies might vary in scope, quality and true biological gaps that could affects results of certain prediction outcomes on different carbon substrate. These limitations in GEMs could be addressed by using gap filling algorithms and strategies described elsewhere [4] depending on the availability of resources and facilities at the researchers’ disposal.

Taken together, and as a proof-of-principle, we demonstrated that *E. coli* GEM can prospectively and effectively identify novel gene deletion target that can increase succinate production from glycerol substrate. The metabolic gene knockout of adhE in *E. coli* under anaerobic condition from glycerol was hypothesized and experimentally confirmed to increase succinate production by nearly 23 fold. As a proof-of-principle, adhE gene was deleted in silico and in wet cell experiment to evaluate the cell’s metabolic succinic production potential from glycerol substrate. The strain constructed (BMS6) in this study would serve as a starting point for systems strategies for industrial strain design and development and could guide future iterative strain design that requires up to ten (10) different steps to achieve industrially relevant titer as proposed by lee and colleagues [34]. The *E. coli* strain BMS6 (Δ*adhE*) is just a starting strain that could accommodate other targets for engineering in the future to achieve target biosynthetic goal of increasing succinate production from glycerol substrate. It is imperative to mention that for proof-of-principle strain such as BMS6 (Δ*adhE*) to achieve industrially relevant titer, additional engineering strategies are required with a great deal of efforts (requiring 50 - 300 persons years of work) and investment (of up to several hundred millions of US dollars) as recently proposed elsewhere [34].

Conflict of Interest

None.

Bibliography

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