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In silico Gene knockout metabolic interventions in *Escherichia coli* for Enhanced Ethanol Production on Glycerol.

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ABSTRACT

Ethanol is one of the leading biofuels which is widely pursued in industry. Systems metabolic engineering, combining computational tools and synthetic microbiology is gaining more attention in enhancing ethanol production by *Escherichia coli* in recent years. However, investigation on metabolic engineering of *E. coli* for increased ethanol production on glycerol, based on gene knockouts simulation using minimization of metabolic adjustment (MOMA) under OptFlux software platform still remained largely unexplored. Here, we show that *in silico* genes knockout encoding for the competing pathway genes such as pyruvate formate lyase (pflA/b0902) and lactate dehydrogenase (ldhA/b1380) under anaerobic conditions in *E. coli* BSKO eliminated lactate production, reduced carbon flux towards acetate in some mutants, and enhanced ethanol flux. The introduced genetic perturbations led to substantial improvement in ethanol flux on glycerol and xylose. We hypothesize that the ability of *E. coli* under anaerobic condition to use other alternative pathways to produce acetyl-CoA, acetate and ethanol is substrate dependent. Furthermore, the choice of substrates for *E. coli* strain re-engineering is extremely important for strain improvement in relation to ethanol productions. Our results demonstrate that the OptFlux software platforms can prospectively and effectively predict metabolic engineering interventions using *E. coli* genome-scale model, based on gene knockout simulation. This would lead to better understanding of *E. coli* systems metabolism for strain re-engineering and improvement.

Key words: *Escherichia coli*, ethanol, metabolic engineering, glycerol, OptFlux and gene knockout simulation

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INTRODUCTION

Ethanol is one of the leading biofuels which is widely pursued in industry [1]. The amount of world ethanol production for transport fuel is continuously increasing and it was forecasted that the global use of biofuel including bioethanol is expected to nearly double in 2017 [2]. Accordingly, there have recently been several reports on the use of *Escherichia coli* systems metabolic engineering for biofuels production [2, 3]. Therefore, systems metabolic engineering, combining advanced computational tools and synthetic biology can provide novel solutions and strategies to further re-engineer *E. coli* metabolic pathways for enhanced ethanol productions.

Glycerol can be considered as a substrate for bioethanol production using *E. coli,* because it has received renewed attention in recent years due to its generation in bulk quantities as a by-product of biofuel industries [4]. Glycerol could be a good substrate to increase ethanol production using *E. coli* as it is not only abundant and inexpensive but also can generate more reducing equivalents than glucose and xylose [4].

Genome scale metabolic reconstructions are of utmost importance as productive tools for their capacity to integrate genomic information to predict desired phenotype and to simulate whole-cell physiology in an interconnected system [5]. Several genome scale metabolic models of *Escherichia coli* have been published recently [6, 7] with ability to reasonably predict accurate growth rates, metabolite excretion rates and growth phenotypes on a number of substrate and genetic condition that is consistent with experimental observation [6, 8].

In addition, it was reported that "the basic and applied uses of genome-scale metabolic reconstruction of *E. coli* have been primarily focused on six (6) applications: (1) metabolic engineering, (2) model driven discovery, (3) prediction of cellular phenotypes, (4) analyses of biological network properties, (5) studies of evolutionary processes and (6) models of interspecies interactions" [9]. However, the understanding of these applications coupled with computational predictive power will expedite forward the *E. coli* systems metabolic engineering for the production of ethanol and other compounds of interest.

The advent of genome-scale metabolic models of *Escherichia coli* has concurrently stimulated the development of several computational tools/software to study *E. coli* systems *in silico.* We previously reported that computational breakthroughs and synthetic microbiology can be synergistically combined to improve strain performance for increase ethanol production [10]. In particular, an open source user friendly computational tool for metabolic engineering applications called OptFlux [11], has been developed and implemented with a genome scale metabolic model of *E. coli* to predict the phenotype simulation of both wild-type and mutant organism, using the method of Flux Balance Analysis (FBA), Minimization of Metabolic Adjustment (MOMA) of Metabolic flux changes [11].

This software platform can be used to computationally predict gene deletions strategies using MOMA simulation for over production of the target compound of interest in *E. coli* stoichiometric model.

However, few studies have reported on the use of computational methods such as OptKnock for studying various gene deletions to increase the production of lactic acid using the *E. coli* stoichiometric model constructed elsewhere [12]. But there is little information available on the use of the OptFlux software platform with *E. coli* genome scale model iAF1260 [6] as a reference metabolic engineering application tool to predict post perturbation effect of competing pathway genes as novel strategies to increase ethanol production in *E. coli*. Although other workers [1, 13] have reported the experimental deletion of certain competing pathway gene(s) / enzymes such as lactate dehydrogenase A (ldhA), pyruvate formate lyase A (pflA) and pyruvate dehydrogenase (pdh) to increase ethanol production in *E. coli*. Their approach is unique as they used different substrate (glucose and gluconate) and imported ethanol production pathway (PET operon) from *Zymomonas mobilis* which becomes integrated in to the pyruvate formate lyase (pflB) locus.

Even though a lot of work has been reported on ethanol production in metabolically engineered *E. coli* strains [2, 4, 14, 15], very little work has been reported on increasing ethanol production in *E. coli* based on gene knockout simulation using the OptFlux software platform. In this study, we investigate whether ethanol flux from glycerol and xylose can be improved, based on *in silico* genes knockout simulation of pyruvate dissimilation pathways (pflA / b0902, ldhA / 1380 and frdBC / b4152, b4153) in *E. coli* stoichiometric model, and how the genetic perturbation (using MOMA) affects the strain performance.

MATERIALS AND METHODS

Model

The work described herein uses the metabolic reconstruction of *Escherichia coli* iAF1260 [6]. This model has been functionally tested and validated against experimental data to be predictive for computations of growth rates, metabolite excretion rates, and growth phenotypes on a number of substrate and genetic conditions [6, 8].

Flux Balance analysis

Flux balance analysis (FBA) and minimization of Metabolic Adjustment (MOMA) were implemented using the Java programming, within the framework of the OptFlux open source platform (http://www.optflux.org) [11]. This provides free user-friendly tools for the metabolic engineering (ME) community aiming to be the reference platform in the field. All simulation of mutant strain and wild type were performed using the OptFlux v3.06.
Substrates (glycerol and xylose) uptake rates for the solitary carbon substrates in each simulation were constrained to a maximum uptake rate of 10 $mmolgDW^{-1}h^{-1}$. For anaerobic simulations the oxygen uptake rate was set to be 0.0 $mmolgDW^{-1}h^{-1}$. This value was chosen based on slightly close experimental observations of anaerobic growth of *E. coli* [24-26]

Gene knockout using the OptFlux software platform

Flux balance analysis predict metabolic flux distributions at steady state by using linear programming [26] whereas minimization of metabolic adjustment (MOMA) employs quadratic programming to identify the point in flux space, which is more or less closest to the wild-type point and consistent with the gene knockout constraint [27]. MOMA was

implemented under the OptFlux software platform as described in their original documentation.

OptFlux simulations using MOMA were run to completion for single and double knockouts strains. The knocks out gene(s) were pflA/b0902, ldhA/b1380 and frdBC/b4152, b4153 BS101 (ΔpflA), BS102 (ΔldhA), BS103 (ΔfrdBC) and BS104 (ΔpflA ΔldhA). The gene knockouts were performed using glycerol and xylose as substrates to increase ethanol production in *Escherichia coli* genome stoichiometric model.

RESULTS

Glycerol as the substrate

When glycerol was used as substrate or carbon source, all the strains are able to ferment glycerol and produce certain amount of ethanol as the main fermentation product with the exception of strain BS103 which lost its ability to grow under anaerobic condition as a result of kocking out frdBC (b4152, b4153). As shown in fig 1, the deletion of pflA (Strain BS101) led to much slower growth rate. The pflA (BS101) single deletion strain maintained only about 6.24% of the wild-type growth rate under anaerobic condition. On the other hand, the growth rate of ldhA (BS102) single deletion strain is about 69.41% of the wild-type growth rate. The double knockout strain BS104 (ΔpflA ΔldhA) led to slightly slower growth rates (fig 2 and table 2) as in strain BS101 (6.24% of the wild-type). There was no growth in strain BS103 as a result of deletion of frdBC.

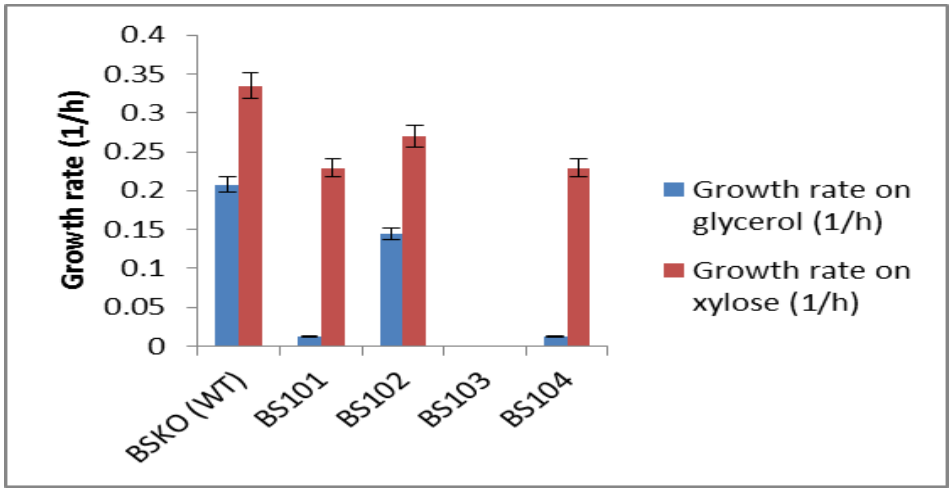

Figure 1: Growth rates of E. coli BSKO and mutant models on glycerol and xylose. Error bars indicate standard deviation on the replicates.

A reasonable amount of ethanol was formed as main fermentation product of *E. coli* BSKO, and all the *in silico* mutant models constructed in this study with the exception of mutant strain BS103. The mutant strains BS101 (ΔpflA) and BS104 (ΔpflA ΔldhA) had a slightly higher ethanol flux (109.42% or 9.42% higher) than the wild-type (see fig 1 and table 2). Other mutants have similar ethanol flux rate as the wild-type (see fig 2). There was no detectable lactate flux produced by the wild-type but a slight lactate was produced by BS101 and BS104 (data not shown). Other by products such as acetate, succinate and

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formate were produced along with ethanol by the wild-type BSKO. All the mutant strains produced slightly less acetate than the wild-type (see fig 2)

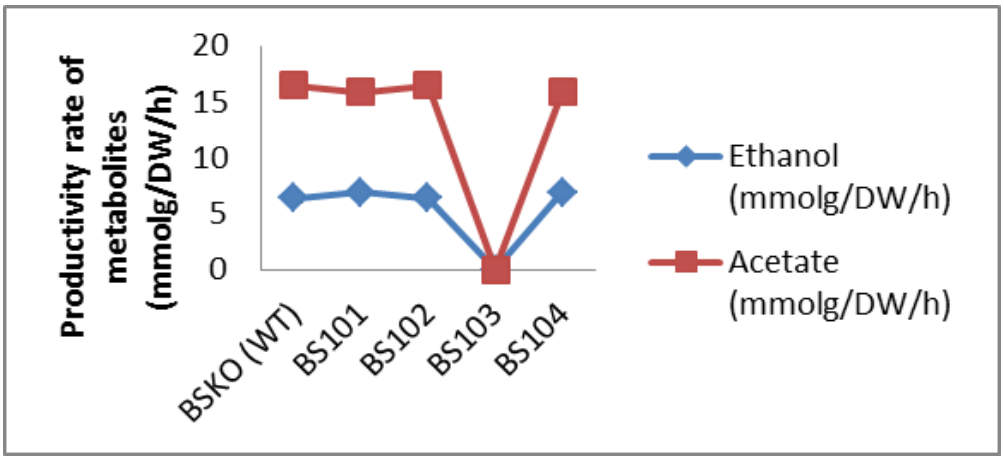

Figure 2: Ethanol and acetate productivity rates of E. coli BSKO and mutant models on glycerol. Error bars indicate standard deviation on the replicates.

Table 1: *E. coli In silico* models used in this study

E. coli strains	Description	Source
E. coli BSKO	Wild-type Model	[6]
BS101	Δ *pflA*	This study
BS102	Δ *ldhA*	This study
BS103	Δ *frdBC*	This study
BS104	Δ *pflA* Δ *ldhA*	This study

Table 2: *E. coli* strain design properties on glycerol under OptFlux software platform

Strains	Knockout genes	Growth rates (1/h)	% Growth rate	Ethanol ($mmolgDW^{-1}h^{-1}$)	% ethanol	Acetate ($mmolgDW^{-1}h^{-1}$)	% acetate
BSKO (WT)	-	0.20783578	100	6.33789	100	16.4721	100
BS101	ΔpflA	0.012968043	6.24	6.9352	109.42	15.85774	96.3
BS102	ΔldhA	0.14426926	69.41	6.34044	100.04	16.47132	99.9
BS103	ΔfrdBC	0.00	0.00	0.00	0.00	0.00	0.00
BS104	ΔpflA ΔldhA	0.012968043	6.24	6.9352	109.42	15.85774	96.3

Maximum uptake rates for glycerol were set to be 10 $mmolgDW^{-1}h^{-1}$ and the corresponding Oxygen uptake rate was 0.0 $mmolgDW^{-1}h^{-1}$ for anaerobic simulation

Xylose as the substrate

Xylose was chosen as the solitary carbon source for *E. coli* BSKO to produce ethanol. When xylose was used, all of the mutants produced ethanol flux in combination with acetate, succinate and formate. The ethanol flux of the wild-type (*E. coli* BSKO) is slightly higher than the acetate (see fig 3 and table 3). Overall, xylose was utilized faster by the strain than glycerol with 61% higher growth rate than when glycerol was used as the substrate (fig 1). Similar trend for growth rates was maintained for all mutants as depicted in Fig 1 and table 3. Single gene knockout strain BS101 (ΔpflA) led to substantially similar

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acetate production as the wild-type (see fig 3 and table 3) with a slight increase in acetate production. However, no lactate was produced. The ethanol flux in strain BS101 was slightly higher (100.08%) than that of the wild-type (see fig 3 and table 3) and exhibited no lactate production. No detectable flux for lactate is realized. The metabolite flux for ethanol in mutants BS101 and BS104 followed the same trend as ethanol flux on glycerol but with a much more increase in acetate production than the wild-type (see fig 3 and table 3)

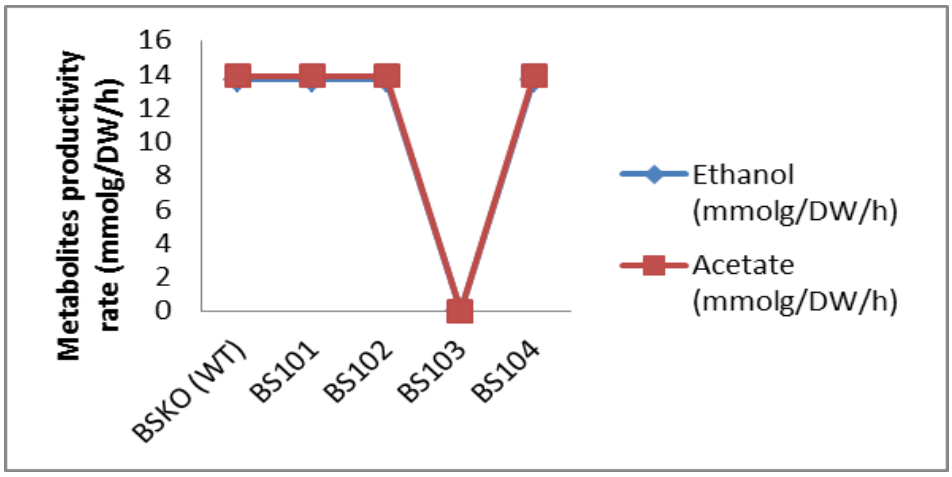

Figure 3: Ethanol and acetate productivity rates of E. coli BSKO and mutant models on xylose. Error bars indicate standard deviation on the replicates.

Table 3: *E. coli* strain design properties on xylose under OptFlux software platform

Strains	Knockout genes	Growth rates (h^{-1})	% Growth rate	Ethanol ($mmolgDW^{-1}h^{-1}$)	% ethanol	Acetate ($mmolgDW^{-1}h^{-1}$)	% acetate
BSKO (WT)	-	0.33500515	100	13.65422	100	13.87054	100
BS101	ΔpflA	0.22882006	68.30	13.66467	100.08	13.8966	100.18
BS102	ΔldhA	0.26959917	80.48	13.65408	99.99	13.87434	100.02
BS103	ΔfrdBC	0.00	0.00	0.00	0.00	0.00	0.00
BS104	ΔpflA ΔldhA	0.22882006	68.30	13.66467	100.08	13.8966	100.18

Maximum uptake rates for xylose were set to be 10 $mmolgDW^{-1}h^{-1}$ and the corresponding Oxygen uptake rate was 0.0 $mmolgDW^{-1}h^{-1}$ for anaerobic simulation

DISCUSSIONS

Esc coli systems metabolic engineering is a powerful approach that can be used to generate renewable compounds such as biofuels for growing human population. Systems biology and in silico analysis have the potential to accelerate the re-engineering of new strains of *E. coli* to increase its production potential through model driven analysis [8]. This work present progress towards this goal through a systematic flux balance analysis (FBA) of the production potential for ethanol from *E. coli* using *in silico* gene knockout simulation strategies under the OptFlux software platform.

Glycerol and xylose are considered to be the two main solitary carbon sources used in this study. As stated earlier there are growing interests in using glycerol as substrate, this is because it becomes abundantly available as a by-product of biodiesel industries [4]. It was

previously established that conversion of glycerol to certain glycolytic intermediates produces twice the amount of reducing equivalents generated by the metabolism of glucose or xylose [4, 15]. Until recently, it was previously thought that *E. coli* was not a suitable organism for fermentative utilization of glycerol since the metabolism of this carbon source required an additional external acceptor [4]. It was previously established that identification of glycerol fermentative pathways in *E. coli* has open up a new horizon to engineer *E. coli* as chassis host for production of ethanol and other various reduced molecules [4, 14, 15]

E. coli carries out mixed acid fermentation under anaerobic conditions and produces acetate, formate, succinate, lactate and ethanol as fermentation products. The deletion of mixed acid fermentation reactions / genes such as frdBC and ldhA have been previously reported [16-18] to increase the intracellular pool of NADH in *E. coli*. It was also previously [17] established that knocking NADH consuming reactions under anaerobic conditions led to completely no growth in *E. coli*, this might be the reason why in silico knocking out of frdBC in this study led to completely no growth in strain BS103 (see fig 1). The mutant strain BS103 (ΔfrdBC) lost its ability to grow anaerobically due to lack of presence of an important NADH-consuming pathway (frdBC / b4152, b4153) as an electron sink (see Fig 4). Such a strain is unable to recycle NADH, thereby creating a driving force for reaction that consume NADH [17, 19, 20]. Three (3) different in silico gene knockout strategies for production of ethanol in *E. coli* genome scale model [6] were carried out using the OptFlux software platform [11]. This framework uses minimization of metabolic adjustment to predict phenotype growth rates after introduction of genetic perturbation (gene knockout) under anaerobic conditions (see materials and methods).

When glycerol was used as a solitary carbon source, ethanol is primarily produced by the wild-type model (BSKO) as well as small amount of acetate under anaerobic conditions (see fig 2, 3 & table 2). The knocking out of pflA in strain BS101 led to production of both ethanol and acetate when glycerol is the substrate (see fig 2 and table 2). The ethanol produced by mutant strain BS101 was higher (9.42%) than that of the wild-type. This might be as a result of the fact that carbon flow to ethanol production is increased as a result of deletion of pflA which is a competing pathway for the production of ethanol (see fig 4). This higher ethanol production by *E. coli* on glycerol has been previously reported elsewhere [4] stressing that the conversion of glycerol to glycolytic intermediates generates twice the amount of reducing equivalents generated by the metabolism of glucose or xylose [4, 21]. As a result, potential yields of ethanol fuels and other chemicals are higher when synthesized from glycerol relative to their monosaccharide counterparts [4, 14]. It was already established that when glycerol was used as carbon source, wild-type *E. coli* produced 86% ethanol and some traces of acetate and lactate [4, 14]. Mutant strain BS101 also produced acetate, despite the deletion of pflA, although the acetate produced is slightly lower than that of the wild-type (see table 2and fig 2). Acetate production is believed to have serious effects on the production of ethanol and other value added compounds when *E. coli* is used as chassis host. This is because more carbon flux is directed toward the production of acetate rather than ethanol, succinate or lactate in *E. coli* central metabolism. Therefore the acetate produced by the wild-type and Mutant strains in this study seems to be primarily from PFL pathway, and in the absence of pflA, the other alternative way for the cell to produce acetyl-CoA is via the pyruvate dehdrogenase (PDH) pathway [1], and/or activation of *pflB* (see Fig 4). *E. coli* pyruvate formate lyase B (pflB), is a central enzyme of

anaerobic metabolism, it catalyzes the co-enzyme A- dependent, non-oxidative cleavage of pyruvate to acetyl-CoA and formate in anaerobically growing cells, its activity is induced under anaerobic conditions [22, 23].

The decrease of acetate production in mutant strain BS101, BS102 and BS104 could increase ethanol production in *E. coli*, as more carbon flux will be directed toward ethanol production or other NADH consuming reactions (succinate and lactate) see fig 4. It was previously reported that the deletion of pflA when glucose was used as substrate in ethanol production using *E. coli* under anaerobic condition, led to no acetate production [1], but in this study in silico deletion of pflA using the OptFlux software platform led to acetate production on glycerol. We can hypothesize that acetate production in *E. coli* under anaerobic condition is substrate dependent. Therefore the choice of enzymes and/or pathways in *E. coli* central metabolism to produce acetate and ethanol could be substrate dependent as well.

The growth rate of the mutant strain BS101 (ΔpflA) and BS104 (ΔpflAΔldhA) on glycerol was slow with 6.24% growth per hour from the wild-type, but with highest ethanol production potential of 9.42% (see table 2). The slow growth rate on glycerol might be attributed to the fact that the hydrolysate is not rich in xylose and mannose which are known to have positive effect on both cell growth and solvent production [4].The challenges of using glycerol as a single substrate still exists, but this could be improved by using co-substrates during fermentation, such as combination of glycerol and xylose or glucose. When xylose was considered as a substrate, a faster growth rate that was 61% higher compared to that of its glycerol counterpart was realized.it was also reported that when other substrates such as xylose or glucose are used in *E. coli*, a faster growth rate is realized because hydrolysate of such substrates is rich in xylose and mannose which are known to have a positive effect on both cell growth and solvent production [4].

When xylose was considered as substrate, in silico deletion of pflA in mutant strain BS101, a slightly higher (100.08%) ethanol flux is achieved (see fig 3 and table 3). This is because we reason that deletion of pflA would decrease the pool of acetate that might favour the carbon flow towards ethanol production in *E. coli.* Surprisingly, Mutant strain BS101 produced slightly more acetate than the wild-type on xylose under anaerobic condition. Normally, when pflA is deleted, the only way for the cell to produce acetyl-CoA and ATP is via PDH pathway and/or activation of pflB under anaerobic conditions [22, 23]. The high acetate produced by mutant strain BS101 in this study, might be attributed to either use of PDH pathway as an alternative route or activation of pflB to generate acetate. On the bases of this findings, we can hypothesize that xylose is the preferred substrate for PDH pathway or pflB. This seems to show how critical the choice of substrate is for *E. coli* strain improvement to increase ethanol production. On other hand, it was reported elsewhere that no acetate was produced after the deletion of PflA in *E. coli* on glucose substrate under anaerobic condition [1] This could be an indication that PDH complex and pflB preferred other substrates like glycerol, xylose and gluconate rather than glucose for activation.

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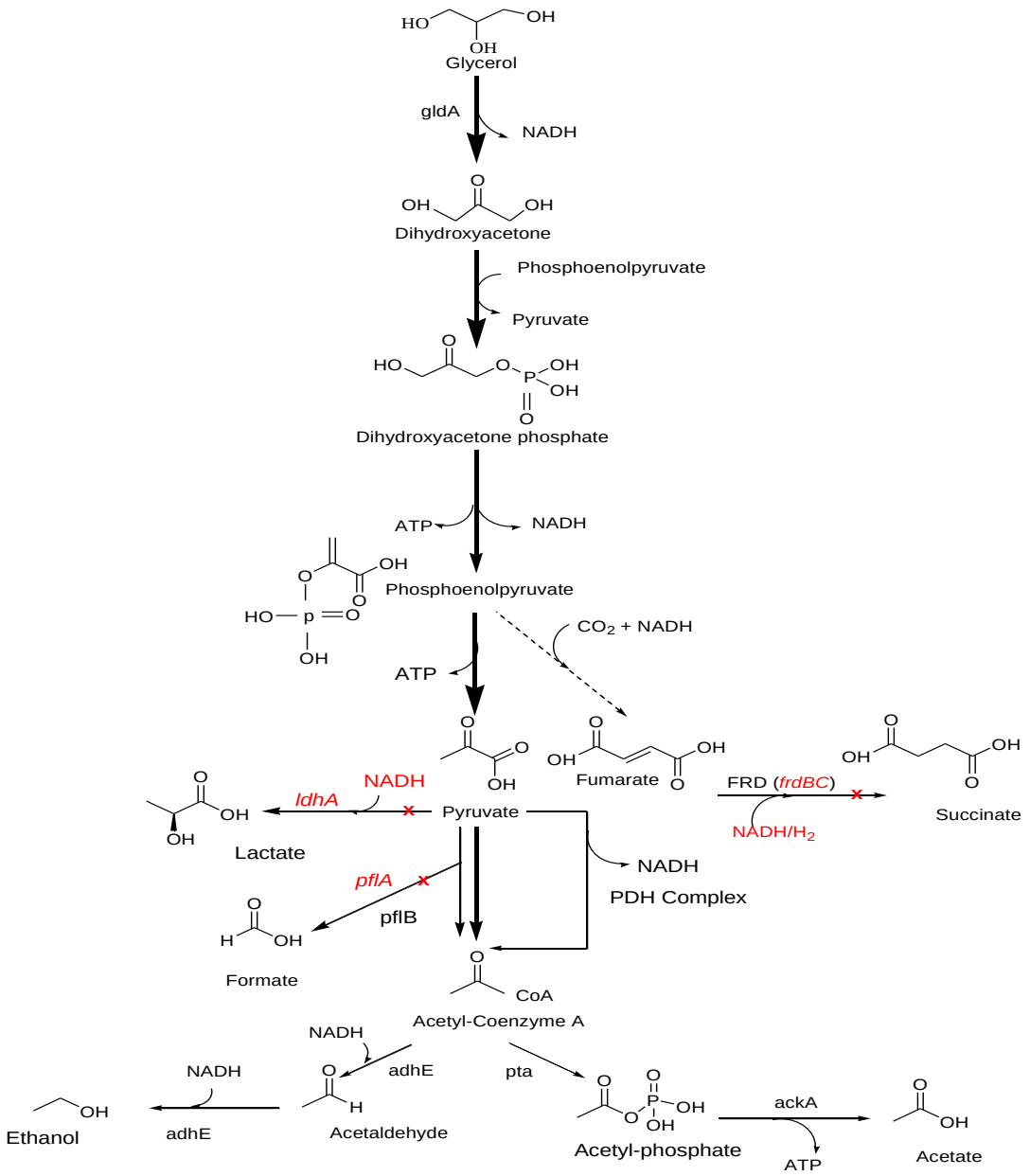

Figure 4: Main fermentative pathways involved in anaerobic fermentation of glycerol in E. coli [partially adopted from [4, 14, 15]]. Pathways involved in fermentative utilization of glycerol by *E. coli* BSKO and its constructed mutant strains. The pathways along with the deleted competing fermentation gene(s) are shown. The enzymes in red represent the pathways that were blocked via gene knockout. The red X indicates deletion, and the NADH in red are consumed during anaerobic fermentation for ethanol production on glycerol. The knockout genes encode for lactate dehydrogenase (ldhA), Pyruvate formate lyase (pflA) and fumarate reductase (frdBC).

CONCLUSIONS

Escherichia coli systems metabolic engineering is a powerful approach that can be used to generate renewable compounds such as biofuels for growing human population. Overall, significant ethanol flux seen in computationally predicted strain of *E. coli* model constructed in this study based on gene knockout simulation using OptFlux software platform proves to be an important approach for strain re-engineering and improvement. Engineered strains of *E. coli* already exist that produced ethanol on different substrates [1,

2, 13-15], and some of the strains constructed here are similar to previously published work [1, 2, 14, 15]. The mutant strains constructed in this study indicate that *pflA* might not be the only enzyme responsible for acetate production in *E. coli* when glycerol and xylose were used under anaerobic condition, while the production of acetate increased more than that of the wild-type even after deletion of pflA on xylose substrate. Alternative pathways such as PDH complex and/or activation of pflB might be responsible for the high acetate production in this in silico studies. We hypothesize and reason that the ability of *E. coli* under anaerobic conditions to use other alternative pathways to produce acetyl-CoA, acetate and ethanol is substrate dependent and that the choice of substrate for *E. coli* strain improvement is critical to achieving high ethanol yield. We finally showed that the OptFlux software platform can prospectively and effectively predict metabolic engineering interventions using *E. coli* genome-scale model, based on gene knockout simulation on glycerol and xylose substrates respectively. Our results also demonstrate that glycerol could be the best substrate for increase ethanol production in engineered *E. coli* strains. These findings would guide future experimental work by allowing us to gain more biological insight and better understanding of *E. coli* systems metabolism for strain re-engineering and improvement to increase ethanol production.

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