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IN SILICO METABOLIC ENGINEERING INTERVENTIONS OF *ESCHERICHIA COLI* FOR ENHANCED ETHANOL PRODUCTION, BASED ON GENE KNOCKOUT SIMULATION

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ABSTRACT

Systems metabolic engineering, combining computational tools and synthetic microbiology is gaining more attention in enhancing ethanol production in Escherichia coli. However, investigation on metabolic engineering of E. coli for increased ethanol production based on genes knockout simulation using minimization of metabolic adjustment (MOMA) under the OptFlux software platform still remained largely unexplored. Here, we show that in silico genes knockout encoding for the competing pathways enzymes like pyruvate formate lyase (pflA/b0902) and lactate dehydrogenase (ldhA/b1380) under anaerobic conditions in E. coli BMKO eliminated lactate production, reduced carbon flux towards acetate, and enhance ethanol flux. The corresponding genetic perturbations led to a small improvement in ethanol flux from 100% to 100.18% on glucose and 100% to 100.08% on gluconate and it is capable of substantial increase. 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. Our results demonstrates that the OptFlux framework can prospectively and effectively predict metabolic engineering interventions using E. coli genome-scale model, based on gene knockout simulation. This would lead not only to better understanding of E. coli systems metabolic engineering, but also could guide future experimental work for strain improvement.

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KEY WORDS

Escherichia coli, ethanol, metabolic engineering, prediction, OptFlux and gene knockout simulation

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[I] 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.

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 [4]. Several genome scale metabolic models of *Escherichia coli* have been published recently [5], [6] 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 [5], [7]

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 [8]. 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 compound of interest.

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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 [9]. In particular, an open source user friendly computational tool for metabolic engineering applications called OptFlux [10], 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) or Regulatory on/off Minimization (ROOM) of Metabolic flux changes [10]. This software platform can also be used to computationally predict gene deletions strategies using either MOMA or ROOM simulation for over production of 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 [11]. But there is little information available on the use of the OptFlux software platform with *E. coli* genome scale model iAF1260 [5] as a reference metabolic engineering application tool to predict post perturbation effects of competing pathway genes as novel strategies to increase ethanol production in *E. coli*. Although other workers [1], [12] 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 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], 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 glucose and gluconate 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 OptFlux software platform can prospectively and effectively predict genetic perturbations in relations to strain performance and ethanol production.

[II] MATERIALS AND METHODS

2.1. Model

The work described herein uses the metabolic reconstruction of *Escherichia coli* iAF1260 [5]. 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 [5], [7]

2.2. Flux balance analysis

Flux balance analysis (FBA) and mininmization of Metabolic Adjustment (MOMA) were implemented using the Java programming , within the framework of the OptFlux open source platform (http://www.optflux.org) [10]. This provides free user-friendly tools for the 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 (glucose and gluconate) uptake rates for the solitary carbon substrates in each simulation were constrained to a maximum uptake rate of10 mmol/gDW/h. For anaerobic simulations the oxygen uptake rate was set to be 0.0 mmol/gDW/h. This value was chosen based on slightly close experimental observations of anaerobic growth of *E. coli* [23, 24, 25].

2.3. Gene knockout using the OptFlux software platform

Flux balance analysis predict metabolic flux distributions at steady state by using linear programming where as minimization of metabolic adjustment (MOMA) employs quadratic programming to identify point in flux space, which is more or less closest to the wild-type point and consistent with the gene knockout constraint [26]. 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 knockout strains. The knockout gene(s) were pflA/b0902, ldhA/b1380 and frdBC/b4152, b4153. The single knockout strains are designated as BMKO1 (ΔpflA or ΔldhA) and the double knock out strains are designated as BMKO2 (ΔfrdBC or ΔpflA ΔldhA) see **Table-1**. The strains were further described as BM101 (ΔpflA), BM102 (ΔldhA), BM103 (ΔfrdBC) and BM104 (ΔpflA ΔldhA). The gene(s) knockout were performed using glucose and gluconate as substrates to increase ethanol production in *Escherichia coli* genome stoichiometric model

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

E. coli strains	Description	Source
E. coli BMKO	Wild-type Model	(Feist et al., 2007; Le Novère et al., 2006)
BM101	BMKO1 Δ*pflA*	This study
BM102	BMKO1 Δ*ldhA*	This study
BM103	BMKO2 Δ*frdBC*	This study
BM104	BMKO2 Δ*pflA* Δ*ldhA*	This study

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[III] RESULTS

3.1. Glucose as the substrate

When glucose was used as the substrate or carbon source, all the strains are able to ferment glucose and produce a reasonable amount of ethanol as the main fermentation product with the exception of strain BM103 which lost its ability to grow under anaerobic condition as a result of knocking out *frdBC* (b4152, b4153). As shown in fig 1, the deletion of *pflA* (Strain BM101) led to slightly lower growth rates. The *pflA* (BM101) single deletion strain maintained about 98% of the wild-type growth rate under anaerobic condition. On the other hand, the growth rate of *ldhA* (BM102) single deletion strain is about 55.58% of the wild-type growth rate, which is the lowest growth rate reported in this study. The double knockout strain BM104 (ΔpflA ΔldhA) led to slightly lower growth rates **[Figure–1 and Table–2]** as in strain BM101 (98% of the wild-type). There is ultimately no growth in strain BM103 as a result of the deletion of the *frdBC* genes.

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

Strains	Knockout genes	Growth rates (1/h)	% Growth rate	Ethanol (mmol/gDW/h)	Acetate (mmol/gDW/h)	% ethanol
BMKO (WT)	-	0.18814504	100	8.495536	8.61685	100
BM101	ΔpflA	0.18466564	98.15	8.51065	0.00	100.18
BM102	ΔldhA	0.10457363	55.58	8.48512	8.61453	99.88
BM103	ΔfrdBC	0.00	0.00	0.00	0.00	0.00
BM104	ΔpflA ΔldhA	0.18466564	98.15	8.51065	8.61147	99.88

Maximum uptake rates for glucose were set to be 10 mmol/gDW/h and the corresponding Oxygen uptake rate was 0.0 mmol/gDW/h for anaerobic simulation

A reasonable amount of ethanol was formed as main fermentation products of *E. coli* BMKO, and all the in silico mutant models constructed in this study with the exception of mutant strain BM103. The mutant strains BM101 (ΔpflA) and BM104 (ΔpflA ΔldhA) had a slightly higher ethanol flux (100.18% or 0.18% higher) than the wild-type **[Figure–1 and Table–2]**. Other mutants have similar ethanol flux rate as the wild-type. There was no detectable lactate flux produced by the wild-type but a slight lactate was produced by BM101 and BM104 (data not shown). Other by-products such as acetate and formate were produced along with ethanol by the wild-type BMKO. No acetate production was observed after deletion of *pflA* (strain BM101). The mutant strains BM102 and BM104 produced slightly less acetate than the wild-type BMKO.

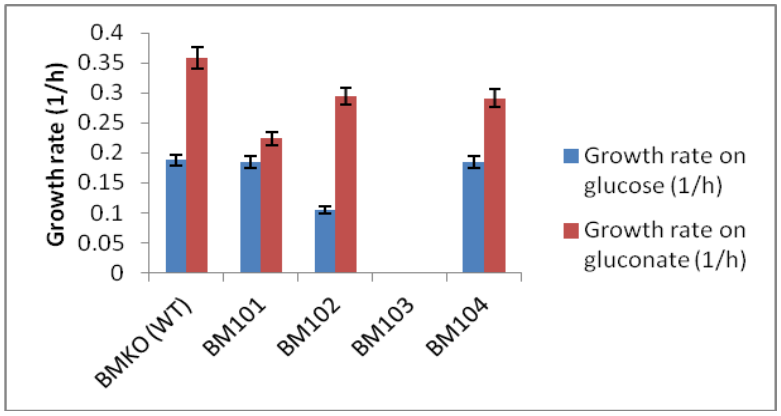

Fig: 1. Growth rates of *E. coli BMKO* and mutants on glucose and gluconate. Error bars indicate standard deviation on the replicates

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3.2. Gluconate as the substrate

Gluconate was chosen as the solitary carbon source for *E. coli* BMKO to produce ethanol. When gluconate 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* BMKO) is slightly higher than the acetate. Overall, gluconate was utilized faster by the strain than glucose with 90% higher growth rate than when glucose is used as the substrate. Similar trend for growth rates was maintained for all mutants as depicted in **Figure– 1 and Table– 3**. Single gene knockout strain BM101 (ΔpflA) led to substantially similar acetate production as the wild-type **[Figure– 3 and Table– 3]** with a slight increase in acetate production. However, no lactate was produced. The ethanol flux in strain BM101 is lower **[Table– 3]** than that of the wild-type and exhibited no lactate production.

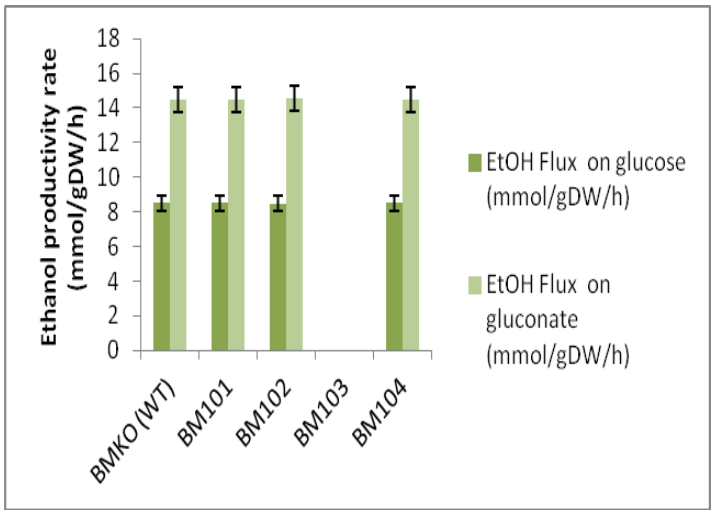

Fig: 2. Ethanol productivity rates of *E. coli BMKO* and mutants on glucose and gluconate. Error bars indicate standard deviation on the replicates

Knocking out *ldhA* (strain BM102) led to increase ethanol flux (100.08%) that is 0.08% high than the wild-type (BMKO) as shown in **Figure– 2 and Table– 3** and only a slightly less acetate (0.045% less) is produced relative to the wild-type **[Figure– 3 and Table –3**). Double knocked out strain BM104 (ΔpflA ΔldhA) does not efficiently direct more carbon flow towards ethanol production [**Figure–2 and Table– 3]**. The mutant strain BM102 (ΔldhA) obtained a slightly higher ethanol flux on gluconate **[Table– 3]** than that of the wild-type (BMKO) with much less acetate production. No detectable flux for lactate is realized.

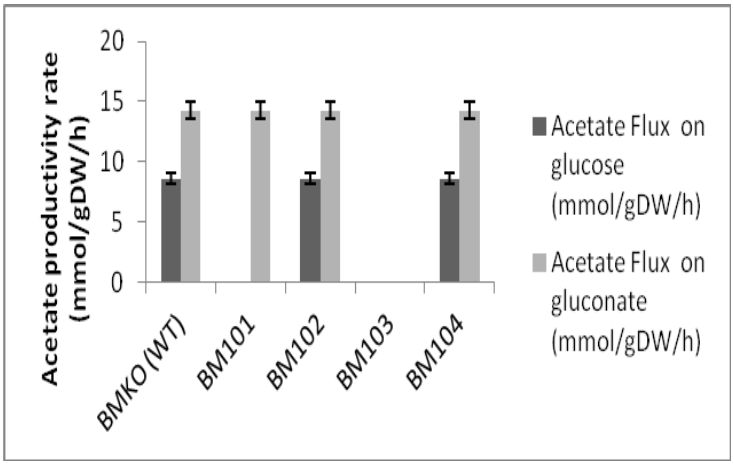

Fig: 3. Acetate productivity rates of *E. coli BMKO* and mutants on glucose and gluconate. Error bars indicate standard deviation on the replicates

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

Strains	Knockout genes	Growth rates (1/h)	% Growth rate	Ethanol (mmol/gDW/h)	Acetate (mmol/gDW/h)	% ethanol
BMKO (WT)	-	0.35828092	100	14.50847	14.24288	100
BM101	ΔpflA	0.22370522	62.44	14.46933	14.25845	99.73
BM102	ΔldhA	0.29466613	83.58	14.52007	14.23636	100.08
BM103	ΔfrdBC	0.00	0.00	0.00	0.00	0.00
BM104	ΔpflA ΔldhA	0.29136069	81.32	14.46933	14.25845	99.73

Maximum uptake rates for gluconate were set to be 10 mmol/gDW/h and the corresponding Oxygen uptake rate was 0.0 mmol/gDW/h for anaerobic simulation.

[IV] DISCUSSION

Eschericha 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 [7]. 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. Gluconate and glucose are considered to be the two main solitary carbon sources.

Eschericha 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 [13-15] to increase the intracellular pool of NADH in *E. coli*. It was also previously [15] 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 BM103**[Figure–1]**. The mutant strain BM103 (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 **[Figure-4].** Such a strain is un able to recycle NADH, thereby creating a driving force for reaction that consume NADH [15–17]. Three (3) different *in silico* gene knockout strategies for production of ethanol in *E. coli* genome scale model [5] were carried out using the OptFlux software platform [10]. 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).

There are four routes for pyruvate dissimilation in *E. coli* KO11: *pfl*, *ldhA pdh* and recombinant PET operon from *Zymomonas mobilis* encoding for pyruvate decarboxylase (pdc) and alcohol dehydrogenase II (adhII) genes [1]. We reason that in silico gene knockout simulation of pyruvate dissimilating enzymes and other competing pathways (encoded by the ldhA/b1380, frdBC/b4152,b4153 and pflA/b0902 genes (without the use of PET operon from *Z. Mobilis*) in *E. coli* genome-scale model could play essential roles in ethanol production.

Under anaerobic conditions, when glucose was used as carbon source, 2 ATP and 2NADH **[Figure–4]** are generated during glycolysis [1]. The required additional ATP per glucose can be realized by dissimilation of pyruvate through PFL pathway **[Figure–4]** which has been demonstrated in *E. coli*, and the increase pool of NADH is used to reduce metabolic intermediates and create fermentative products [1, 18]. When a more oxidized substrate like gluconate is used, only 1 ATP and 1 NADH are generated through the Entner-Doudoroff pathway **[Figure–4]**. The consequence of this oxidized substrate necessitate the cell to produced more oxidized products such as acetate, lactate, formate succinate and ethanol as an alternative means to balance the accumulating NADH [1, 19]. It was theoretically established that 1 mole of gluconate is required to produced 0.5 moles of acetate, 1. 5 moles of ethanol and 1.5 ATP whereas 1 mole of glucose is required to produced 2 mole of ethanol and 2 ATP [1, 12]. On the other hand the growth rate of the mutant strain BM101 (ΔpflA) is about 98% of the wild-type (BMKO) under anaerobic condition, while that of the mutant strain BM102 (ΔldhA) is about 55.5% of the wild-type

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(considered as marginal growth) **see Figure–1**and **Table–2**. This seems to clearly indicate the significance of *ldhA* in the growth rate of *E. coli* mutant strain on glucose under anaerobic conditions. It could utilized only a little glucose under anaerobic conditions because of the lack of major anaerobic respiratory pathways[20]

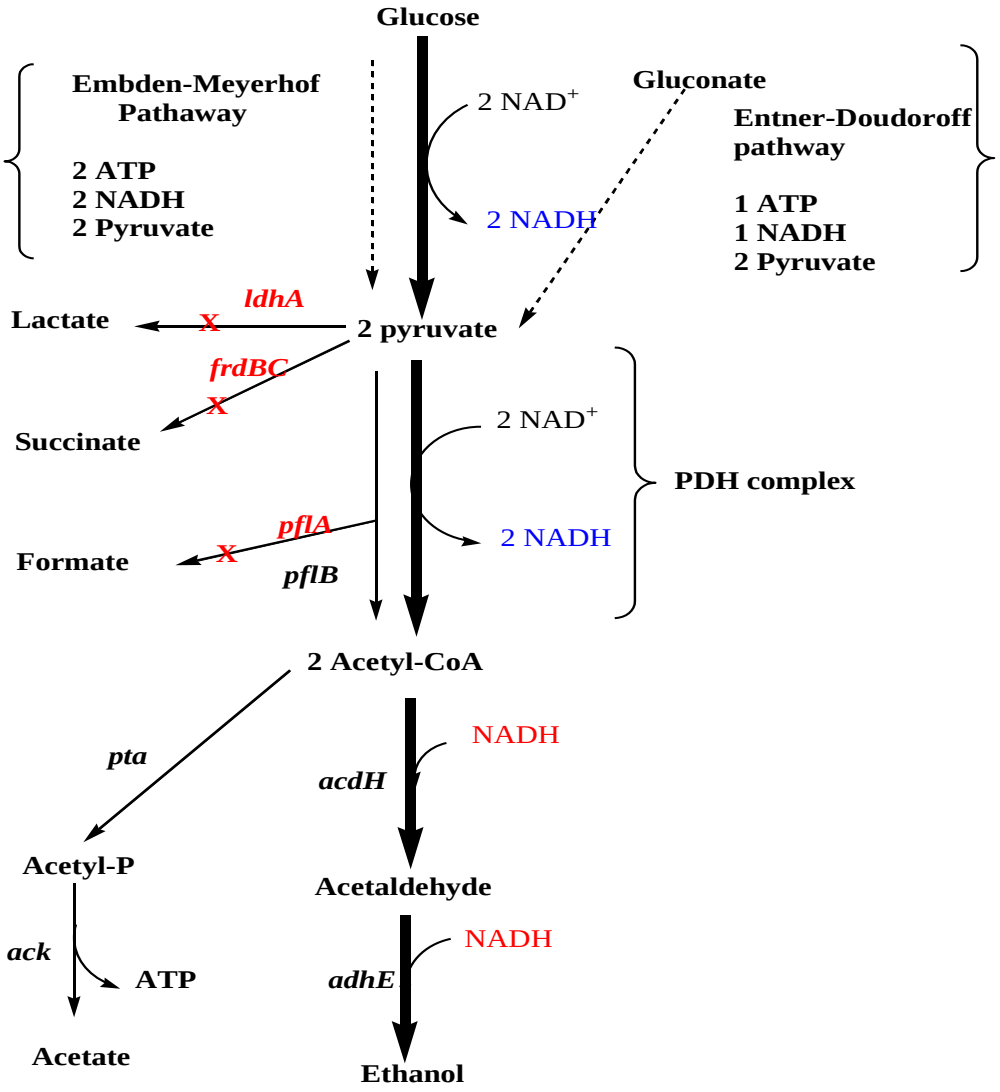

Fig: 4. Pathways involved in fermentative utilization of glucose and gluconate by *E. coli* BMKO and its constructed mutant strains. The pathways along with the deleted competing fermentation pathways are shown. The enzymes in red represent the pathways that were blocked via gene knockout. The knockout genes encode for lactate dehydrogenase (ldhA), Pyruvate formate lyase (pflA) and fumarate reductase (frdBC)

When glucose was used as a solitary carbon source, ethanol is primarily produced by the wild-type model (BMKO) as well as small amount of acetate **[Figure–2, -3 and Table–2)**. The knocking out of *pflA* in strain BM101 led to no acetate production when glucose is the substrate, whereas strain BM101 on gluconate produced acetate in combination with ethanol fermentative products **[Figure-3]**. 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. The inability of strain BM101 to produced acetate *in silico* using glucose as substrate is in agreement with a work previously reported elsewhere [1], where deletion of *pflA* in *E. coli* KO 11 totally eliminated the acetate production. Therefore the acetate produced by wild-type BMKO 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 PDH pathway [1], and/or activation of *pflB* **[Figure-4]**. *E. coli* pyruvate formate lyase B

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(*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 [21, 22].

The decrease and/or absence of acetate production 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). The flux to ethanol in mutant strain BM101 (ΔpflA) and BM104 (ΔpflAΔladhA) was slightly higher than the wild-type **[Table– 2, 3 and Figure–2]**. We can hypothesize that mutant strain BM101 can produce certain amount of ethanol in combination with acetate as fermentation products **[Figure–2, 3 and Table– 3]** when gluconate was used as substrate, while on the hand the same strain BM101, produced ethanol and other fermentation products but without formation of acetate when glucose was used as the substrate **[Table– 2]**. This might be as a result of the fact that gluconate is more oxidized than glucose and in addition, the cell might have used the alternative pathways to produce acetyl-CoA and acetate through the PDH pathway and/or *pflB* is induced to produce acetate under anaerobic condition with gluconate as susbtrate. Pyruvate formate lyase (*pflB*), has been previously reported [20, 21] to be induced under anaerobic condition, but yet no acetate was produced in mutant strain BM101 (ΔpflA) when glucose was the substrate under the same condition. We can hypothesize that anaerobicity is not the only factor that is critical to induction of *pflB* activity, this is because strain BM101 (ΔpflA) produced acetate under anaerobic condition with gluconate as substrate. The activity of *pflB* seems to be substrate dependent, stressing the need for careful selection of substrate to reduce acetate formation and increase ethanol production in *E. coli*.

When gluconate was used as substrate, mutant strain BM101 (ΔpflA) lead to substantially similar acetate production as the wild-type **[Figure– 3 and Table– 3]** with a slight increase in acetate production. The ethanol flux was only 0.08% higher than that of the wild-type on gluconate. Surprisingly, no lactate was produced. This *in silico* result is contrary to the findings reported elsewhere [1], where the mutant strain deficient of *pflA* exhibited an increase in lactate production, although their Mutant strain had a foreign gene designated as PET operon on its *pflB* locus from *Z. Molbilis*.

Double knockout strain BM104 (ΔpflA/ΔldhA) does not seem to efficiently direct carbon flow towards ethanol production **[Figure–2 and Table– 2, -3]**. This result is contrary to the work reported previously [1], where double knockout strain (ΔpflA ΔldhA) demonstrated the highest ethanol production on both glucose and gluconate substrates respectively. 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 [21, 22], in such circumstances excess NADH will be generated and thus, can only be re-oxidized to NAD^+ through the native ethanol pathway or exogenous PET operon from *Z. Mobilis* [1]. The fact that the double knockout strain BM104 of this study is deficient of exogenous PET operon from *Z. Mobilis*, could be the reason why it cannot produce higher ethanol on both glucose and gluconate substrates. The nutant strain BM102 (ΔldhA) obtained a slightly higher ethanol flux (0.08% higher) on gluconate from the wild type (BMKO) with much less acetate production. Since the acetate production was slightly lower than the wild-type (BMKO), we can hypothesize that flux through PFL is responsible for the increase acetate production. It was previously reported that PFL appears to be the preferred route of acetyl-CoA generation over the PDH pathway when the ldhA was knockout [1]

[IV] CONCLUTION

Systems metabolic engineering, combining computational tools and synthetic microbiology is gaining more attention in enhancing ethanol production in *E. coli*. Overall, significant ethanol flux seen in computationally predicted strain of *E. coli* model constructed in this study based on gene knockout simulation using the OptFlux software platform proves to be an important approach for strain improvement. While engineered strain of *E. coli* already exist that produce ethanol [1, 2, 12] and some of the strain constructed here are similar to previously published work [1, 2]. The mutant strains constructed in this study indicates that *pflA* might be responsible for acetate production in *E. coli* when glucose was used under anaerobic condition, while the production of acetate when gluconate was used under the same condition might be as a result of activation of *pflB* and/or pyruvate dehydrogenase complex. We hypothesize and reason that the ability of *E. coli* under anaerobic condition to use other alternative pathways to produce acetyl-CoA, acetate and ethanol is substrate dependent. 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, and it could guide future experimental work rather than relying solely on the use of the conventional experimental trial by error approach.

CONFLICT OF INTEREST

Author declares no conflict of interest.

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