Biosci Bioeng Commun 2015; 1: 1-10

Bioscience and Bioengineering Communications

Journal Home: https://www.journalbinet.com/

Model-guided *in silico* deletion of *pntA* gene predicts increased succinate production under anaerobic conditions in *Escherichia coli*

Bashir Sajo Mienda and Mohd Shahir Shamsir

Bioinformatics Research Group (BIRG), Department of Biosciences & Health Sciences, Faculty of Biosciences and Medical Engineering, Universiti Teknologi Malaysia, Johor Bahru, Malaysia

Article History

Received 29 Jun 2015
Revised 10 Jul 2015
Accepted 15 Jul 2015
Online 20 Aug 2015

Key words

E. coli genome scale model, gene knockout simulation, OptFlux software, prediction, pyridine nucleotide transhydrogenase, and succinate

ABSTRACT

The production of succinic acid under anaerobic conditions in Escherichia coli faces a major obstacle of NADH limitation. Here, we used *E. coli* genome scale model and engineered *in silico* gene knockout strategies by deletion of the membrane-bound pyridine nucleotide transhydrogenase (*pntA*) using Minimization of Metabolic Adjustment (MOMA) algorithm with the OptFlux software platform. The metabolic role of this transhydrogenase during *E. coli* bio-catalysis in relation to succinic acid production has remained largely unspecified. This study informs other studies that the in silico deletion of this gene predicts increase succinic acid production in *E. coli*. The results indicate that the simulation of the mutant model lacking *pntA/*b1603 under anaerobic conditions with glucose as the substrate predicts increased succinate production that is twofold higher than the wild-type parent model. On the bases of these findings, we hypothesize that knocking out of the *pntA* transhydrogenase in E. coli might have resulted in a twofold increase in NADH availability, that catalyzes enhanced succinate production. These findings open up a novel model-driven biological inquiry in determining the underground metabolic function of the *pntA*/b1603 in NADPH and/or NADH regeneration in relation to succinic acid production in E. coli.

Corresponding author: Bashir Sajo Mienda**,** Bioinformatics Research Group (BIRG), Department of Biosciences & Health Sciences, Faculty of Biosciences and Medical Engineering, Universiti Teknologi Malaysia, Johor Bahru, Malaysia. Email: bashirsajomienda@fbb.utm.my

INTRODUCTION

Succinate is currently considered to be a valuable molecule, and it has a specialty chemical market, in the industrial production of food, agricultural and pharmaceutical products, surfactants and detergents [1]. It can also serve as a precursor for the synthesis of commodity chemicals used in plastics and solvents, with an estimated potential global market of $15 billion [2]. Other molecules of interest that can be chemically derived from succinic acid by known chemical processes include: 1,4-butanediol, maleic anhydride, succinimide, 2 pyrolidonone and tetrahydrofurans [3]. A large market exists for these molecules and they can be readily converted into varieties of molecules for use in polymers, industrial solvents and biodegradable succinate esters [3]. As a result of the aforementioned applications, succinic acid is currently placed on a list of top twelve platform chemicals from biomass by the US department of energy (DOE) [3].

Escherichia coli is preferred for succinic acid production, because it is considered to be a user friendly chassis host and a well characterized microorganism with deep understanding of its genetics and physiological regulations as opposed to other hosts [4]. Although *Saccharomyces cerevisiae* could be readily applied, but additional metabolic pathway engineering to re-wire the chassis host must be required because it is not a natural over producer of succinic acid. *E. coli* has the advantages of faster growth rates, facultative anaerobes, non-spore forming bacterium allowing great avenues for flexibility and cost effective process design for large scale industrial production of the target compound of interest [5]. *E. coli* has been engineered to produce succinate using different carbon sources [6-11]. In addition, several *E. coli* strains were previously constructed in which competing pathway genes among others, were knocked out and no foreign genes were expressed [3,12]. Several rounds of metabolic evolution approach were applied using this strategy, by selecting strains with improved growth in minimal medium with glucose as the main carbon source [3] .The outcome of this strategy produced *E. coli* strains with improved capabilities for succinate production [12].

The increase in modern genome sequencing facilities and capabilities lead to the reconstruction of a biochemical reaction network called Reactome to build *in silico* metabolic models [13]. These metabolic models, particularly of *E. coli* developed previously [14-16] help in guiding metabolic engineering targets. The models of *E. coli* with ability of predicting phenotype – genotype relationship have been tested and validated against established experimental data [17-19]. It was reported to have a number of applications, including model driven biological discovery, metabolic engineering, and prediction of cellular phenotypes and analysis of biological network properties among others [19]. With increasing reconstructions of genome scale metabolic model and accuracy in *in silico* simulation, the model has now been considered as an indispensable tool to design and rewire microbial metabolism for stream improvements [20]. Comparative genome approach with *in silico* gene knockout simulation using MetaFluxNet was reported

for increased succinate production in *E. coli* [7]. A more recent studies on bilevel optimization technique in computational strain redesign using the OptKnock and the OptForce as the prototype has been reported [21]. The challenge of using the OptKnock technique for prediction of metabolic gene knockout exists, as it only allowed reaction deletions instead of gene knockouts in genome scale metabolic models [22,23]. This is because reactions deletions could be difficult to be implemented in a wet lab experimental setting, as the real organism may contain isoenzyme, protein complexes and enzyme that catalyze several reactions, which could lead to false positive predictions[22,23]. In contrast, the use of the OptFlux software platform for *in silico* metabolic engineering intervention for increased ethanol production from glucose [24] and glycerol [25] have been demonstrated, which allowed successful *in silico* gene knockout [24]. Furthermore, *in silico* metabolic gene knockout has been evaluated for increased D-lactate production from glycerol with the OptFlux software platform [23].

E. coli has two isoforms of transhydrogenases designated as *UdhA* (encoding a soluble pyridine nucleotide transhydrogenase) [26,27] and *pntAB* (encoding for a membrane-bound pyridine nucleotide transhydrogenase) [27] The two isoforms have unknown physiological functions but could potentially transfer electrons directly from NADH to $NADP^+$ and vice versa [27]. It was established by Fischer and co-workers that proton trans-locating transhydrogenase *pntAB* as a major source of NADPH in *E. coli* under aerobic glucose growth condition [27]. It can be reasoned that *pntA* is involved in oxidoreductase activity involving the two currency metabolites (NADH and NADPH), and because the two isoforms have been established to have a divergent physiological function: reduction of $NADP^+$ with NADH by *pntAB* via energy dependent mechanism and oxidation of NADPH by *UdhA* [27]. In a similar study by Hummel and co-workers [28], whole cell biocatalyst was constructed, that contained a recombinant alcohol dehydrogenase (R-ADH), Formate dehydrogenase (FDH) and pyridine nucleotide transhydrogenase (PNT) for the efficient regeneration of NADPH in relation to the conversion of acetophenone to (R)-) henylethanol [28]. Furans tolerance in ethanol producing *E. coli* was increased by introducing a plasmid encoding NADPH/NADH transhydrogenases (*pntAB*) as described previously [29]. In this study, we used the knowledge based created by the aforementioned studies to couple the *in silico* deletion of *pntA* gene in *E. coli* for increased succinic acid production. This is because it has been established that 2 molecules of NADH is required to make 1 molecule of succinate in *E. coli* under anaerobic condition [30]. In addition, it was also reported elsewhere [28] that *pntA* can be used to catalyze the transfer of hydrogen from NADH to $NADP^+$ (see reaction 1) The current study aims to understand for the first time how the deletion of the membrane-bound *pntA* gene might affect certain metabolic pathways that could be primarily responsible for supplying additional NADH to the fumarate reductase, as a step toward increasing the pool of NADH availability for enhanced succinic acid production in *E. coli*.

We reason that by deleting the membrane-bound *pntA* transhydrogenase in *E. coli* (see Fig 2), increased pool of NADH under anaerobic condition could be established (see reaction 1), thereby creating additional NADH for the mutant cell to produce more succinic acid relative to the wild-type strain (see reaction 2).

We first combined stoichiometric metabolic network modeling with knowledge of NADH regeneration by *in silico* deletion of membrane-bound *pntA* transhydrogenase under anaerobic condition to understand how this enzyme knockout influence the succinate production by *E. coli*. Mutant model was then created to investigate the contribution of particular pathways (see Fig 2) or reactions (see reaction 1 and 2) toward succinic acid yield. We found that the mutant model lacking *pntA/b1603* constructed herein, has a predicted improved succinate production that is twofold higher compared to the wild-type control model.

MATERIALS AND METHODS

Modelling and computational tool

The metabolic reconstruction of *E. coli* K-12 MG16655, designated as iJO1336, was used for all simulations of the mutant and the wild-type models in this work [16] Flux balance analysis (FBA) and Minimization of Metabolic Adjustment (MOMA) were implemented in the OptFlux software platform www.optflux.org [31] as previously described in their original documentations [31,32]. All the simulations conducted in this study were implemented using the Java programming in the OptFlux software interface as described elsewhere[31].

The chosen solitary carbon source is glucose unless otherwise stated. For each simulation the carbon source was constrained to a maximum uptake rate of 18.5 mmol gDW^{-1} h^{-1}. For the anaerobic simulation, the oxygen uptake rate was set to be a maximum of 0.0 mmol gDW^{-1} h^{-1}. These values were selected based primarily on established experimental observations for *E. coli* growth under anaerobic condition as described previously[33-37].

In silico analysis of gene deletion

Gene knockout simulation was performed with the OptFlux interface by using the Minimization of Metabolic Adjustment (MOMA) algorithm as previously described [32]. MOMA is an algorithm for flux based analysis, which uses quadratic programming for searching the nearest point in the feasible solution space of a designated mutant in relation to the optimal point in the feasible solution space of the wild-type control model [32,38]. The mutant model constructed in this study with the deletion of the *pntA* is designated as BMRK (Δ*pntA/b1603*), while the wild-type control model is designated as BMR (WT Orth Model).

RESULTS AND DISCUSSION

The wild-type *E. coli* produces a mixture of fermentation products, including formate, ethanol, acetate and negligible amount of succinate under anaerobic conditions, whereas under aerobic condition succinate is only produced as an intermediate of the tricarboxylic acid cycle (TCA) when the glyoxalate by pass is operating [3]. The

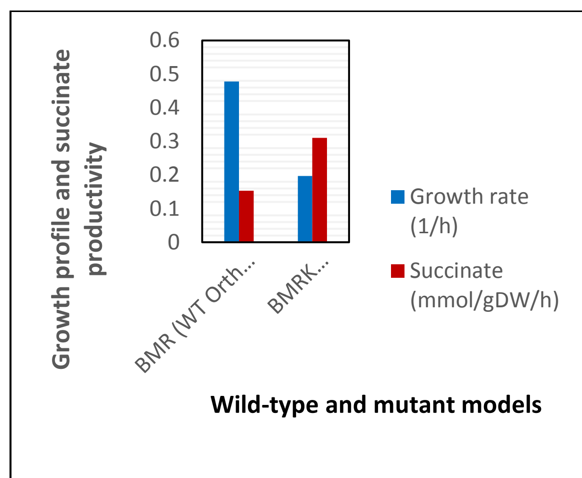


Fig 1: *In silico* growth profile and succinate productivity of the wild-type and mutant models from glucose substrate.

maximum theoretical yield of succinate in wild-type *E. coli* is 1 mol/mol glucose in anaerobic conditions; this yield is limited by reducing the equivalent availability (NADH). In this study, we used genome scale metabolic reconstruction of *E. coli* *i*JO1336 to investigate the role of the *in silico* deletion of membrane bound transhydrogenase (*pntA/b1603*) in increasing the availability of NADH for enhanced succinic acid production in *E. coli*. The role of *pntA* in a batch aerobic glucose growth has been reported to be the major source of NADPH in *E. coli*[27]. We took the advantage that the role of the enzyme (*pntA*) is particularly important in aerobic glucose growth than anaerobic growth in *E. coli*, then we performed *in silico* deletion of the gene to increase NADH availability in relation to succinate production under anaerobic conditions (see Fig 2). The expression of the soluble (*UdhA*) and membrane bound (*pntA*) transhydrogenases was hypothesized to be modulated by the redox state of cellular metabolism, because any manipulation in genetic and/or environmental conditions that increased or decreased NADPH availability down regulated *pntA* and *UdhA* transcription respectively[27].

The *in silico* deletion of *pntA* in *E. coli* leads to increased succinate production from glucose, but with an impaired growth rate that is 41.2% of the wild-type control model (BMR) under anaerobic condition (see table 1 and Fig 1). The problem of growth rate of the mutant strain under anaerobic conditions can be resolved by applying what is described as “dual phase” fermentation approach in a real experimental setting as previously established [8,39]. This strategy was shown to be successful by growing the mutant strains under aerobic conditions for biomass generation and subsequently switching to anaerobic condition for succinate production[8,39].

The model-guided increase succinate production by the deletion of *pntA* reached about 202% of the wild-type control model (see Table 1 and Fig 1). This could be attributed to the fact that the conversion of the generated NADH to NADPH has been hampered by the deletion of membrane-bound (*pntA/b1603*) transhydrogeanse (see reaction 1). It was reported previously that *pntA* transhydrogenase is involved in oxidoreductase activity involving the two currency metabolites (NADH and NADPH) for increase xylitol production in *E. coli* (see reaction 2) [40]. The molecules of NADH might have been increased under anaerobic condition following the deletion of the *pntA*.

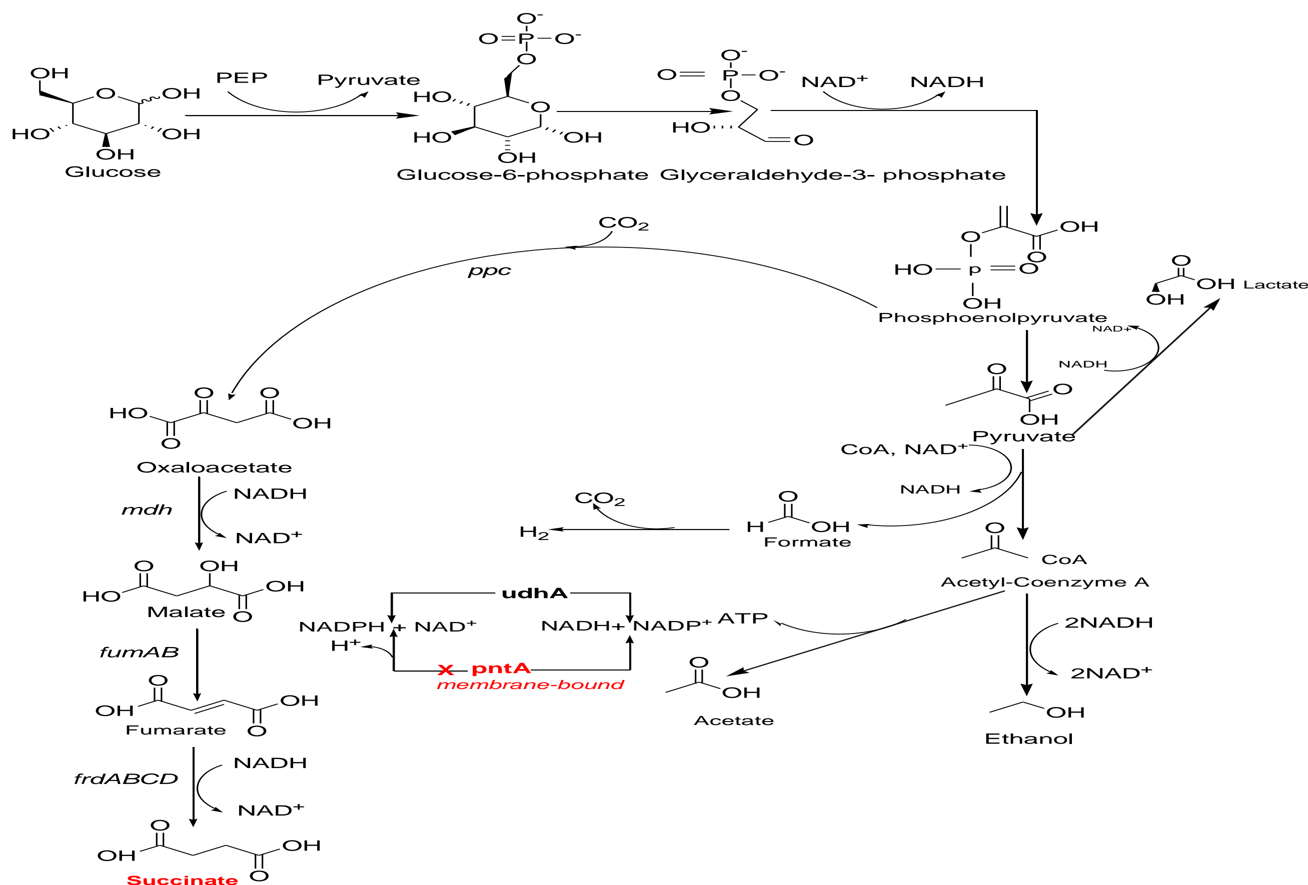


Fig 2: Normal fermentative Pathways for succinate production in *E. coli* under anaerobic condition using glucose as the substrate. Mixed acid fermentation products (ethanol, acetate, formate, lactate and succinate) produced by anaerobic glucose growth of *E. coli* are shown, partially adopted from ref: [3]. The TCA cycle does not function. The proposed deleted (knocked out) membrane-bound pyridine nucleotide (*pntA/b1603*) transhydrogenase is shown in red, partially adopted from ref: [27]. The target compound, succinate is shown in red. Abbreviations: *pntA*, membrane bound pyridine nucleotide transhydrogenase; *ppc,* phosphoenolpyruavte carboxylase; *mdh*, malate dehydrogenase; *fumAB*, fumarse isozymes; and *frdABCD*, fumarate reductase.

Table 1: ***E. coli*** **model design properties with the OptFlux software platform using glucose as substrate**

E. coli model	Knockout genes	In active Reactions	Biomass (h^{-1})	% Biomass	Succinate (mmol g DW^{-1} h^{-1})	% succinate	Acetate (mmol g DW^{-1} h^{-1})	Ethanol (mmol g $DW^{-1}h^{-1}$)
BMR (WT Orth Model)	WT	WT	0.47772028	100	0.15362	100	15.00003	14.72246
BMRK (ΔpntA/b1603),	(ΔpntA/b1603	R_THD2pp	0.19696488	41.2	0.31088	202	14.58916	14.81476

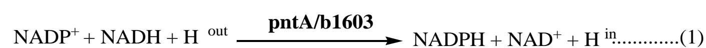
Maximum uptake rates for glucose were set to be 18.5 mmol gDW^{-1} h^{-1} and the corresponding Oxygen uptake rate was zero for anaerobic simulation.

It was established that for the native production of 1 molecule of succinate by *E. coli* under anaerobic conditions, 2NADH are consumed [30]. In principle, under anaerobic condition, succinate is the H-acceptor in lieu of oxygen, the reductive branch partition of

TCA cycle is therefore used [30] The accumulation of succinate emanates from the phosphoenolpyruvate (PEP), through certain intermediate compounds of TCA reductive branch, such as oxaloacetate (OAA), malate and fumarate (see Fig 2 and reaction 2) [30]. The production of succinate was achieved by the used of the pathway from oxaloacetate, malate, fumarate and finally to succinate (see Fig 2). The wild-type model produced 0.15362 mmol gDW^{-1} h^{-1} of succinate (considered as 100%), while on the other hand the mutant model produced 0.31088 mmol gDW^{-1} h^{-1} (considered to be a twofold increase relative to the wild-type model). Based on these findings, the results of the *pntA* deletion seem to indicate that twice the number of reducing equivalent (NADH) could have been generated, thereby increasing the succinate production capability to twofold from the wild-type control model. We can now hypothesize that the deletion of membrane-bound *pntA/b1603* in *E. coli* under anaerobic condition using glucose as the substrate increased NADH availability, thereby creating a driving force for the reaction that facilitates increased succinic acid production.

CONCLUSIONS

The metabolic role of membrane-bound *pntA* transhydrogenase during *E. coli* biocatalysis in relation to succinic acid production has not yet been elucidated. In this study, we report for the first time the *in silico* deletion of the *pntA* transhydrogenase predicts increase succinate production in *E. coli* genome scale metabolic model, and that increased NADH availability might have contributed to the twofold increase in succinate production in the mutant model compared to its wild-type counterpart. To this end, the likely reason for increased succinate production after the deletion of *pntA* transhydrogenase has been unfolded, enabling a significant role of the model-driven experimental inquiry and/or novel biological discovery of the underground metabolic function of this enzyme in relation to NADH regeneration coupled with succinate production in *E. coli*.



Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

The study was supported by Department of Biosciences & Health Sciences, Faculty of Biosciences and Medical Engineering, Universiti Teknologi Malaysia, Johor Bahru, Malaysia.

REFERENCES

1 Zeikus, J. G., Jain, M. K. & Elankovan, P. Biotechnology of succinic acid production and markets for derived industrial products. *Appl Microbiol Biotechnol* **51**, 545 - 552 (1999).

2 Jarboe, L. R. *et al.* Metabolic Engineering for Production of Biorenewable Fuels and Chemicals: Contributions of Synthetic Biology. *Journal of Biomedicine and*

Biotechnology **2010**, 1-18, doi:10.1155/2010/761042 (2010).

3 Chandresh Thakker, Irene Martínez, Ka-Yiu San & Bennett, G. N. Succinate production in Escherichia coli. *Biotechnology journal* **7**, 1-12, doi:10.1002/biot.201100061 (2013).

4 Atsumi, S. *et al.* Metabolic engineering of Escherichia coli for 1-butanol production. *Metabolic Engineering* **10**, 305-311, doi:http://dx.doi.org/10.1016/j.ymben.2007.08.003 (2008).

5 Atsumi, S., Hanai, T. & Liao, J. C. Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels. *Nature* **451**, 86-89, doi:10.1038/nature06450 (2008).

6 Chatterjee, R., Millard, C. S., Champion, K., Clark, D. P. & Donnelly, M. I. Mutation of the ptsG gene results in increased production of succinate in fermentation of glucose by Escherichia coli. *Appl Environ Microbiol* **67**, 148-154, doi:10.1128/AEM.67.1.148-154.2001 (2001).

7 Lee, S. J. *et al.* Metabolic engineering of Escherichia coli for enhanced production of succinic acid, based on genome comparison and in silico gene knockout simulation. *Appl Environ Microbiol* **71**, 7880-7887, doi:10.1128/aem.71.12.7880-7887.2005 (2005).

8 Lin, H., Bennett, G. N. & San, K. Y. Metabolic engineering of aerobic succinate production systems in Escherichia coli to improve process productivity and achieve the maximum theoretical succinate yield. *Metabolic engineering* **7**, 116-127, doi:10.1016/j.ymben.2004.10.003 (2005).

9 Wang, Q., Chen, X., Yang, Y. & Zhao, X. Genome-scale in silico aided metabolic analysis and flux comparisons of Escherichia coli to improve succinate production. *Applied microbiology and biotechnology* **73**, 887-894, doi:10.1007/s00253-006-0535-y (2006).

10 Zhang, X., Shanmugam, K. T. & Ingram, L. O. Fermentation of glycerol to succinate by metabolically engineered strains of Escherichia coli. *Appl Environ Microbiol* **76**, 2397-2401, doi:10.1128/AEM.02902-09 (2010).

11 Förster, A. H. & Gescher, J. Metabolic engineering of Escherichia coli for production of mixed-acid fermentation end products. *Frontiers in Bioengineering and Biotechnology* **2**, 1-12, doi:10.3389/fbioe.2014.00016 (2014).

12 Jantama, K. *et al.* Combining metabolic engineering and metabolic evolution to develop nonrecombinant strains of Escherichia coli C that produce succinate and malate. *Biotechnology and bioengineering* **99**, 1140-1153, doi:10.1002/bit.21694 (2008).

13 Monk, J. & Palsson, B. O. Genetics. Predicting microbial growth. *Science* **344**, 1448-1449, doi:10.1126/science.1253388 (2014).

14 Jennifer L Reed, Thuy D Vo, Schilling, C. H. & Palsson, B. O. An expanded genome-scale model of Escherichia coli K-12 (iJR904 GSM/GPR). *Genome Biology* **4** (2003).

15 Feist, A. M. *et al.* A genome-scale metabolic reconstruction for Escherichia coli K-12 MG1655 that accounts for 1260 ORFs and thermodynamic information. *Molecular systems biology* **3**, 121, doi:10.1038/msb4100155 (2007).

16 Orth, J. D. *et al.* A comprehensive genome-scale reconstruction of Escherichia coli metabolism--2011. *Molecular systems biology* **7**, 535, doi:10.1038/msb.2011.65 (2011).

17 Oberhardt, M. A., Palsson, B. Ø. & Papin, J. A. Applications of genome-scale metabolic reconstructions. *Molecular systems biology* **5**, doi:10.1038/msb.2009.77 (2009).

18 Feist, A. M. *et al.* Model-driven evaluation of the production potential for growth-coupled products of Escherichia coli. *Metabolic engineering* **12**, 173-186, doi:10.1016/j.ymben.2009.10.003 (2010).

19 McCloskey, D., Palsson, B. O. & Feist, A. M. Basic and applied uses of genome-scale metabolic network reconstructions of Escherichia coli. *Molecular systems biology* **9**, 661, doi:10.1038/msb.2013.18 (2013).

20 Xu, C. *et al.* Genome-scale metabolic model in guiding metabolic engineering of microbial improvement. *Appl Microbiol Biotechnol* **97**, 519-539, doi:10.1007/s00253-012-4543-9 (2013).

21 Chowdhury, A., Zomorrodi, A. R. & Maranas, C. D. Bilevel optimization techniques in computational strain design. *Computers & Chemical Engineering* **72**, 363-372, doi:10.1016/j.compchemeng.2014.06.007 (2015).

22 Vilaça, P., Rocha, I. & Rocha, M. A computational tool for the simulation and optimization of microbial strains accounting integrated metabolic/regulatory information. *Bio Systems* **103**, 435-441, doi:10.1016/j.biosystems.2010.11.012 (2011).

23 Mienda, B. S., Shamsir, M. S. & Salleh, F. M. In silico metabolic engineering prediction of Escherichia coli genome model for production of D-lactic acid from glycerol using the OptFlux software platform. *International Journal of Computational Bioinformatics and In Silico Modeling* **3**, 460-465 (2014).

24 Mienda, B. S., Shamsir, M. S., Shehu, I., Deba, A. A. & Galadima, I. A. In silico metabolic engineering interventions of escherichia coli for enhanced ethanol production, based on gene knockout simulation. *IIOABJ* **5**, 16-23 (2014).

25 Mienda, B. S. & Shamsir, M. S. In silico Gene knockout metabolic interventions in Escherichia coli for Enhanced Ethanol production on Glycerol. *RES J PHARM BIOL CHEM SCI* **5**, 964-974 (2014).

26 BOONSTRA, B., FRENCH, C. E., WAINWRIGHT, I. & BRUCE, N. C. The udhA Gene of Escherichia coli Encodes a Soluble Pyridine nucleotide transhydrogenase. *Journal of bacteriology* **181**, 1030-1034 (1999).

27 Sauer, U., Canonaco, F., Heri, S., Perrenoud, A. & Fischer, E. The soluble and membrane-bound transhydrogenases UdhA and PntAB have divergent functions in NADPH metabolism of Escherichia coli. *The Journal of biological chemistry* **279**, 6613-6619, doi:10.1074/jbc.M311657200 (2004).

28 Weckbecker, A. & Hummel, W. Improved synthesis of chiral alcohols with Escherichia coli cells co-expressing pyridine nucleotide transhydrogenase, NADP+-dependent alcohol dehydrogenase and NAD+-dependent formate dehydrogenase. *Biotechnology letters* **26**, 1739–1744 (2004).

29 Miller, E. N., Turner, P. C., Jarboe, L. R. & Ingram, L. O. Genetic changes that increase 5-hydroxymethyl furfural resistance in ethanol-producing Escherichia coli LY180. *Biotechnology letters* **32**, 661–667, doi:10.1007/s10529-010-0209-9.

30 Cheng, K.-K., Wang, G.-Y., Zeng, J. & Zhang, J.-A. Improved Succinate Production by Metabolic Engineering. *BioMed Research International* **2013**, 1-12, doi:10.1155/2013/538790 (2013).

31 Rocha, I. *et al.* OptFlux: an open-source software platform for in silico metabolic engineering. *BMC systems biology* **4**, 45, doi:10.1186/1752-0509-4-45 (2010).

32 Segre, D., Vitkup, D. & Church, G. M. Analysis of optimality in natural and perturbed metabolic networks. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 15112-15117, doi:10.1073/pnas.232349399 (2002).

33 Varma, A., Boesch, B. W. & Palsson, B. O. Stoichiometric interpretation of escherichia-coli glucose catabolism under various oxygenation rates. *Applied and Environmental Microbiology* **59**, 2465-2473 (1993).

34 Lewis, N. E. *et al.* Omic data from evolved E. coli are consistent with computed optimal growth from genome-scale models. *Molecular systems biology* **6**, 390, doi:10.1038/msb.2010.47 (2010).

35 King, Z. A. & Feist, A. M. Optimizing Cofactor Specificity of Oxidoreductase Enzymes for the Generation of Microbial Production Strains—OptSwap. *Industrial Biotechnology* **9**, 236-246, doi:10.1089/ind.2013.0005 (2013).

36 Edwards, J. S., Ibarra, R. U. & Palsson, B. O. In silico predictions of Escherichia coli metabolic capabilities are consistent with experimental data. *Nature biotechnology* **19**, 125-130, doi:10.1038/84379 (2001).

37 VARMA, A. & PALSSON, B. Stoichiometric Flux Balance Models Quantitatively Predict Growth and Metabolic By-Product Secretion in Wild-Type Escherichia coli W3110. *Appl. Environ. Microbiol* **60**, 3724-3731 (1994).

38 Kim, H. J. *et al.* Genome-wide analysis of redox reactions reveals metabolic engineering targets for D-lactate overproduction in Escherichia coli. *Metabolic engineering* **18**, 44-52, doi:10.1016/j.ymben.2013.03.004 (2013).

39 Vemuri, G. N., Eiteman, M. A. & Altman, E. Effects of Growth Mode and Pyruvate Carboxylase on Succinic Acid Production by Metabolically Engineered Strains of Escherichia coli. *Applied and Environmental Microbiology* **68**, 1715-1727, doi:10.1128/aem.68.4.1715-1727.2002 (2002).

40 Chin, J. W., Khankal, R., Monroe, C. A., Maranas, C. D. & Cirino, P. C. Analysis of NADPH supply during xylitol production by engineered Escherichia coli. *Biotechnology and bioengineering* **102**, 209-220, doi:10.1002/bit.22060 (2009).