In silico prediction of *Escherichia coli* metabolic engineering capabilities for 1-butanol production

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***In silico* metabolic engineering interventions have received renewed attention due to the increase in the number of annotated genomes and the development of several genome-scale metabolic models. Using the retrosynthetic metabolic pathway prediction method, we engineered metabolic strategies for the production of 1-butanol by *Escherichia coli* using the OptFlux software platform. The metabolic engineering model shows that the insertion of nucleotide sugar dehydrogenase enzyme from *Leptothrix cholodnii* is predicted to catalyse the production of 1-butanol in *E. coli*. The growth rate and the secretion profile of the mutant model was retained as the wild-type. The result demonstrates that the proposed engineered strain is capable of substantial butanol production increase when 1-butanol gene (*nsdh/b3544*) is overexpressed under semi-anaerobic conditions with fixed glucose and oxygen uptake rates of 8 mmol g DW^{-1} h^{-1} and 5 mmol g DW^{-1} h^{-1}, respectively. We anticipate that our *in silico* results would serve as a starting point for novel *in vivo* metabolic engineering strategies of 1-butanol production in *E. coli*.**

Keywords: Butanol, *Escherichia coli*, metabolic engineering, OptFlux, prediction and retrosynthesis.

A discipline that deals with designing microbial production strains such as *Escherichia coli*, having enhanced capabilities to produce certain compounds of interest, is called metabolic engineering (ME)[1]. This field has received extraordinary attention in the last few years, due to increased growth in the adoption of white or industrial biotechnological processes to produce bulk chemicals and biofuels among other products[2] and their availability to the public. There has been a boost in *in silico* ME strategies with genome-scale metabolic models (GSMM)[3–5] using open source software platform such as OptFlux. The GSMM has become an indispensable tool because of its applications in *in silico* simulation for predicting model-guided ME and synthetic biology strategies for microbial strain improvement[6]. The application of GSMM in guiding microbial strain improvements (e.g. *E. coli*) focusing primarily on a systematic approach and its significant achievements in various industrial fields has been extensively reviewed[6].

Increasing interests in the production of 1-butanol from non-petroleum-derived feed stocks, especially biomass is an important deriver for the construction of new recombinant chassis host such as *E. coli* for these purposes. ME as a discipline; has provided a framework that can be integrated with new computational tools and methodologies for construction of *de novo* design pathways[7]. A *de novo* approach for biosynthesis involves the combination of partial pathways from discrete species and the use of engineered or promiscuous enzymes for the extensions of pathways, specifying biosynthetic routes[7]. This approach is analogous to the practices of retro synthesis used by organic chemists to specify a synthesis scheme towards a target compound, which can be used or applied to specify biosynthetic pathways. Therefore, the approach of retrosynthetic biology is an interesting area to explore to produce 1-butanol in *E. coli.*

The absence of relevant pathways for advanced fuel production in *E. coli* and the opportunity paved by metabolic engineering to produce non-traditional biofuels through the construction of non-native biosynthetic pathways[8] has been of particular interest for 1-butanol production in *E. coli.* As an alternative biofuel, 1-butanol has been considered attractive with high energy density and favourable compatibility with potential to completely replace gasoline. Normally, *Clostridium acetobutylicum* produces 1-butanol together with acetone, butyrate and ethanol[9]. However, the absence of genetic tools and the bacterium's complex physiology has limited the ability to improve it to a level that application in a modern industrial process is feasible[5,9]. As the knowledge of 1-butanol construction in *E. coli* has become available[5], it is now feasible to reconstruct the butanol pathway in *E. coli.* In an example by the same group[5], six sets of genes (*thl*, *hbd*, *crt*, *bcd adh*E2) from *C. acetobutylicum* were successfully transferred to *E. coli* and mixed acid fermentation reactions were deleted to increase butanol titre. This engineered strain produced up to 552 mg l^{-1} of 1-butanol from a rich medium under semi-anaerobic conditions. In another recent study[2], the homobutanol fermentation pathway was established by cloning butanol pathway genes from *C. acetobutylicum* and regenerating 4 NADH require to produce one molecule of butanol by deleting all the NADH consuming pathways genes/reactions (Figure 1 *a*), but only butanol titre of 1254 mg l^{-1} was realized under anaerobic condition. While the product titre still remains low, a novel way for butanol production in a non-native host has been considered a significant achievement. In an attempt to achieve higher titre and productivity, two key genes involved in 1-butanol

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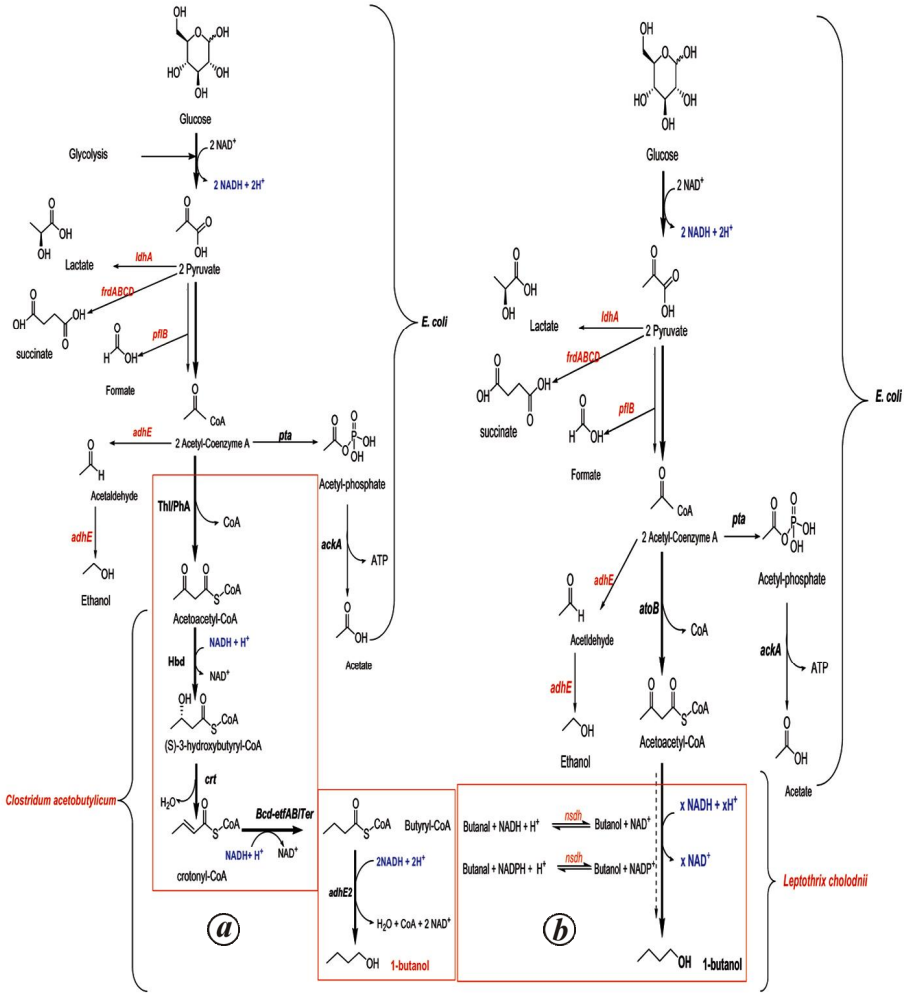

Figure 1. ***a***, Traditional butanol fermentative pathway engineered into *E. coli* from *Clostridium acetobutylicum*, containing six (6) enzymatic steps and requiring 4 NADH to make one molecule of butanol, adapted from refs 2, 4 and 5. ***b***, Proposed novel hypothetical predicted reaction from *Leptothrix cholodnii* for 1-butanol production in *E. coli* using the concept of retrosynthetic biology. It has only one enzymatic step.

pathway have been substituted and their effects explored[4]. Putative NADH-independent butyryl-CoA dehydrogenase replacement with irreversible NADH-dependent transenoyl-CoA reductase and in turn replacement of *Clostridium* acetoacetyl-CoA thiolase with *E. coli* acetyl-CoA acetyltransferase, along with mixed acid fermentation reaction gene deletions, resulted in a strain that produced 30 g l^{-1} butanol with 70–88% of the theoretical yield when grown aerobically followed by anaerobic fermentation[9]. To this end, the 1-butanol fermentative pathway genes were cloned into *E. coli* for increased production. Thus, researchers have exploited the initial experimental trial and error approach to import the 1-butanol fermentative pathway from *C. acetobutylicum* into *E. coli*, yet only the highest titre of 30 g l^{-1} has been achieved to date.

An alternative strategy to engineer 1-butanol production in *E. coli* is to reconstruct the *de novo* biosynthetic pathway using RetroPath prediction webserver coupled with *in silico* metabolic engineering strategies. Non-native enzymes such as nucleotide sugar dehydrogenase from *Leptothrix cholodnii* could be predicted to catalyse 1-butanol production in *E. coli.* These non-native enzymes may be more efficient than enzymes modified using protein engineering[10]. A computer-aided design (CAD) for metabolic engineering using RetroPath and the concept of retrosynthetic biology has been extensively reviewed previously by Faulon and co-workers[11]. This concept was adopted in this research.

Scientific interest shown in the *E. coli* modification to increase butanol availability and yield seen in the aforementioned strains suggests that a computational approach to model 1-butanol production in *E. coli* could guide future experimental work and ultimately affect metabolic engineering. Determining non-native enzymes that could catalyse 1-butanol production reactions, and which

enzymes or genes are affected during modifications are likely to have the greatest impact on the system, and the question of how these changes can be paired with reaction knockouts most effectively, can be answered with an *in silico* modelling procedure. There are a number of studies demonstrating clearly how *in silico* metabolic engineering strategies, combining computational tools with GSMM and synthetic biology could revolutionize the industrial production of biochemicals and biofuels by microbial strain redesign and engineering[6]. In addition, we reported elsewhere, the *in silico* metabolic engineering interventions for enhanced ethanol production on glucose[12] and glycerol[13] substrates respectively. Similar studies reported *in silico* evaluation of gene knockout candidates for D-lactate production in *E. coli* that is consistent with established experimental data[14].

As a tool for investigating metabolic engineering targets *in silico*, OptFlux software, is a metabolic engineering platform that has been shown to enable accurate prediction of bacterial behaviour under different conditions[1,15]. OptFlux utilizes GSMMs, which are built by pairing the constructed metabolic networks of an organism with governing constrained based on physicochemical conservations and/or reaction stoichiometric and environmental parameters. Maranas and co-workers, developed a graph-based algorithm for the identification of non-native routes and *in silico* engineering interventions for 1-butanol production in *E. coli*[16]. Their approach used already established Clostridium butanol fermentative pathway in *E. coli* as previously reported by Atsumi[5,8]. Similarly, Trinh[17], recently reported the use of elementary mode (EM) analysis to reprogram the native fermentative metabolism of *E. coli* for optimized anaerobic production of *n*-butanol and isobutanol through a number of gene knockouts (8–9 genes), additions of multiple genes (6–7 genes), up and down regulation of some genes (6–7 genes) and cofactor (NADH and NADPH) engineering. In contrast to the aforementioned findings, this study finds that a single heterologous pathway gene/enzyme from *L. cholodnii* has been predicted to catalyse 1-butanol biosynthesis in *E. coli* (Figure 6 *b*), opening a novel platform for *de novo* biosynthetic pathway engineering approach using the concept of retrosynthetic biology[11,18]. Until now, investigation on the use of retrosynthetic biology approach (RetroPath) and *in silico* metabolic engineering strategies for 1-butanol production using *E. coli* GSMM remained largely unexplored. In this study, we report for the first time the following: (i) A retrosynthetic biology approach is used to predict 1-butanol production in *E. coli*. (ii) An enzyme called nucleotide sugar dehydrogenase (*nsdh*) has been predicted to catalyse 1-butanol production in *E. coli*. (iii) The imported pathway gene for 1-butanol biosynthesis is from *L. cholodnii* not *C. acetobutylicum* as previously known. The mutant *E. coli* models constructed in this study were found to be capable of substantial increase in butanol production. This will serve as a starting point for *in vivo* metabolic engineering strategies for 1-butanol production in *E. coli*.

Pathway prediction was performed using the RetroPath webserver strictly following the method described previously[11,18]. In this study, *E. coli* was chosen as the chassis host while 1-butanol was selected as the target compound of interest. The reaction signature of height $h = 6$ was selected among myriad options of the RetroPath. This method uniquely among others first tested for thermodynamic feasibility, enzyme performance and homogeneity. Estimation of gene compatibility and norminal flux of the overall pathway was also considered using this approach.

The *i*AF1260 metabolic construction of *Escherichia coli* str. K12 substr. MG1655 was used for simulation in this study[19]. The effect of the addition of predicted gene/reaction from the KEGG database was evaluated using the OptFlux software platform[1].

Flux balance analysis (FBA) and parsimonious flux balance analysis (pFBA) were implemented using Java programing, within the framework of the OptFlux open source platform (http://www.optflux.org)[1]. This provides free user-friendly tools for the ME community aiming to be the reference platform in the field. All simulation of mutant and wild type models were performed using the OptFlux ver. 3.06.

Substrate (glucose) uptake rates for solitary carbon substrates in each simulation were constrained to a maximum uptake rate of 10.0 mmol g DW^{-1} h^{-1}. For aerobic simulations, the oxygen uptake rate was set to a maximum of 18.5 mmol g DW^{-1} h^{-1}. These values were chosen based on slightly close experimental observations of aerobic and anaerobic growth of *E. coli*[20–22].

The OptFlux software platform was used to add 1-butanol as non-native metabolite to the stoichiometric model. The reaction was predicted using the RetroPath webserver with KEGG ID of R03544 and R03545 balanced stoichiometrically and subsequently added to the *E. coli* model. This was designated as *E. coli* BSM101 ($nsdh^+$) mutant model (Table 1).

These reactions were predicted to catalyse 1-butanol production and enzyme involved is called *nsdh* which is present in *L. cholodnii*

$$\text{Butanal} + \text{NADH} + \text{H}^+ \overset{nsdh}{\rightleftharpoons} \text{Butanol} + \text{NAD}^+,$$

$$\text{Butanal} + \text{NADPH} + \text{H}^+ \overset{nsdh}{\rightleftharpoons} \text{Butanol} + \text{NADP}^+. \quad (1)$$

In the above reaction, only butanol is not endogenous to *E. coli* in the stoichiometric model used in this study. The metabolite was added to the model using OptFlux ver. 3.06 (Figure 2). Both reactions and 1-butanol metabolite were added to the *E. coli* wild type model[19] obtained from a biomodel database[3] and hence designated as mutant model used in this study.

Table 1. Sources and characteristics of *E. coli* model strains used

Models	Relevant genotype or phenotype	Reference
Feist model	Wild-type	19
BSM1011	Δ*adhE* + *nsdh*$^+$	This study
BSM1012	Δ*ldhA* + *nsdh*$^+$	This study
BSM1013	Δ*frdBC* + *nsdh*$^+$	This study
BSM1014	Δ*adhE* Δ*ldhA* Δ*frdBC* + *nsdh*$^+$	This study
BSM101	Feist model + *nsdh*$^+$ over expressed	This study
BSM102	Δ*adhE* Δ*ldhA* Δ*frdBC* + *nsdh*$^+$ over expressed	This study

Abbreviations: Alchol dehydrogenase *E* (*adhE*), lactate dehydrogenase *A* (*ldhA*), fumarate reductase *BC* (*frdBC*) and nucleotide sugar dehydrogenase (*nsdh*). Δ: Deleted enzymes; +: Added enzyme.

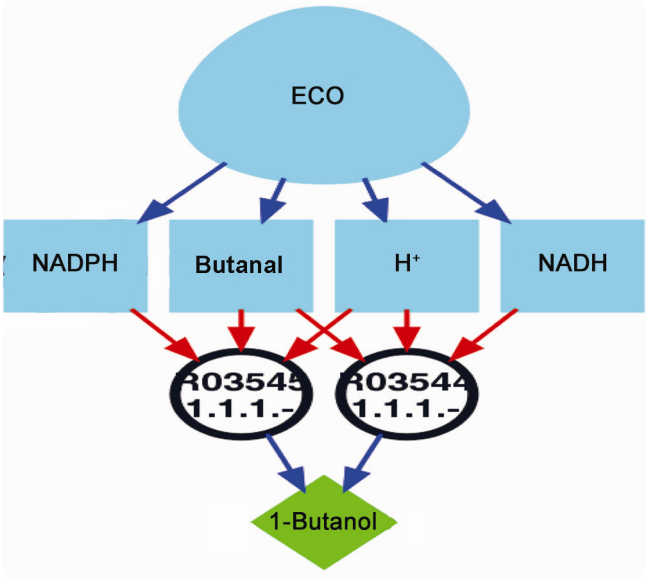

Figure 2. Retrosynthetic map for the production of 1-butanol in *E. coli*. Compounds such as NADPH, butanal, H^+ and NADH are indigenous to the chassis organism (*E. coli*); enzymatic reactions are represented as circles and the target compound is at the bottom of the plot.

Sensitivity analysis of *E. coli* BSM101 mutant model was performed according to the method described previously[19]. Briefly, sensitivity analysis conducted in this study used aerobic environmental condition with limited glucose uptake rate in minimal medium. The parameter being investigated was varied for each analysis, while the glucose uptake rate (GUR) was set between 0 and 10 mmol g $DW^{-1}h^{-1}$ for a series of simulations with their maximum oxygen uptake rate (OUR) set to 18.5 mmol g DW^{-1} h^{-1}. This chosen maximum uptake rate closely matched the maximum uptake rate of oxygen observed *in vivo*[20–22]. In this approach, estimation of maintenance parameters, physiological predictions of growth rate and secretion profile were strictly emphasized. Simulation was conducted as described above and the results were recorded as appropriate.

The mixed acid fermentation reaction (ethanol, lactate and succinate) genes in *E. coli* mutant model were deleted *in silico* to establish an artificial driving force that would facilitate high titre 1-butanol production as described elsewhere[4]. Briefly, alcohol dehydrogenase (*b1241/adhE*), fumarate reductase (*b4153*, *b4152/frdBC*) and lactate dehydrogenase (*b1380/ldhA*) were deleted, and the mutant model carrying these three deletions is designated as BSM102 (*nsdh*$^+$ Δ*ldhA*$^-$ Δ*frdBC*$^-$ and Δ*adhE*$^-$) (Table 1). Simulations were performed using the gene knockout functions of the OptFlux ver. 3.06. Minimization of metabolic adjustment (MOMA) was selected as the simulation method as described previously[23,24]. Growth rates of the mutant models were evaluated using a fixed glucose uptake rate of 8.0 mmol g DW^{-1} h^{-1} under aerobic (oxygen = 18.5 mmol g DW^{-1} h^{-1}), semi-anaerobic (oxygen = 5.0 mmol g DW^{-1} h^{-1}) and anaerobic (oxygen = 0.0 mmol g DW^{-1} h^{-1}) conditions in the presence and/or absence of mixed acid fermentation reactions genes stated here.

The nucleotide sugar dehydrogenase gene designated as *nsdh/b3544*, *b3545* was added and overexpressed to a maximum expression level of 4 for BSM101 and BSM102 (Table 1) using the OptFlux ver. 3.06 as described previously[15]. Behaviour of the mutant models was evaluated using the method of parsimonious flux balance analysis (pFBA) as described elsewhere[25].

A bio-retrosynthetic approach was used to predict the feasibility of importing a novel *de novo* metabolic pathway into *E. coli* as a chassis host to evaluate cost-effectiveness of non-fermentative production of high titre butanol. *E. coli* among other industrial strains was chosen as the chassis organism because of its peculiar feature of being a facultative anaerobe, its fast growth rates with unique flexibility to genetic modification and adaptability to economical process design for large-scale production[8]. In order to find a trade-off between the inherent complexity of *de novo* pathway design and the use of experimental information, it is appropriate to adopt a retrosynthetic method developed previously[18], a tool based on coding of compound and reactions through molecular signature, which is characterized by varying heights *h*. The retrosynthetic graph generated at signature $h = 6$ showed the feasibility of producing 1-butanol successfully, with the major currency metabolite (NADH and NADPH) as indigenous substrate to the chassis *E. coli* host (Figure 2), while on the other hand, reactions catalysed by exogenous

Table 2. Rank list of pathways

ID	Cost	n_genes	Putative	Compound (C06142)	Reaction	r cost	Best_gene	g cost
8265	0.72	1	0	R03545_sscan6_0	R03545	0.00	lch:Lcho_0641	0.72
8264	0.72	1	0	R03545_sscan6_0	R03544	0.00	lch:Lcho_0641	0.72

Each row corresponds to the insertion of one enzyme in the pathway in order to produce the given intermediate product (C06142) in the fifth column. The reaction cost (r cost) and the gene cost (g cost) are given in the seven and ninth columns respectively, and the overall pathway cost is given in the second column. The number of gene (n gene) involved in the pathway is given in the third column, while the best gene to engineer, identified as nucleotide sugar dehydrogenase (*nsdh*) from the KEGG database is given in column eight. KEGG reaction ID of the predicted gene is given in column six.

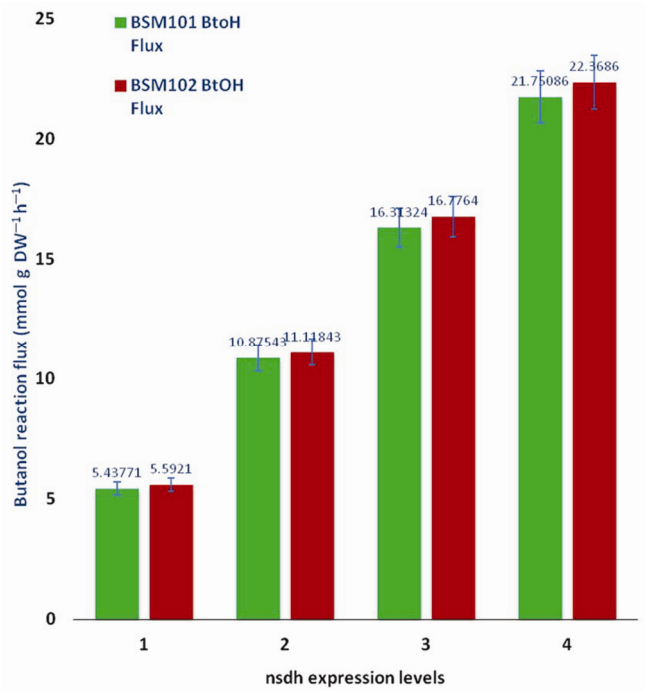

Figure 3. Butanol reaction flux in the mutant models with and without competing pathway genes. BSM101 (WT model + *nsdh* gene). BSM102 (Δ*ldhA* Δ*adhE* Δ*frdBC* + *nsdh* gene). Results represent the means of three determinations. Error bars indicate variation from the mean that is not more than 5%.

enzyme 'nucleotide sugar dehydrogenase' have been predicted to catalyse 1-butanol production.

Metabolic reaction signatures have been considered as the basis of this prediction, and are, usually given by the difference between the signatures of the product and substrate. The signature coding system was made more or less specific to the target compound and reactions by selecting the height *h*. In this study, the height was chosen to be 6. Low heights are less specific as molecular signatures become more and more ambiguous, while higher height values are more specific, as molecular signature has become increasingly precise. As such, the numbers of *de novo* reactions and ultimately *de novo* metabolic pathways can be controlled[18].

Results of the RetroPath prediction indicate the cost of insertion to each pathway based on several criteria such as gene insertion cost, enzyme efficiency, expression levels and nominal flux. Efficiency ranking function of the RetroPath webserver applied in this study gives a good opportunity to select the heterologous pathway(s) to engineer in the chassis *E. coli*. The identified pathway(s) contained both known biochemical transformations that were previously reported as well as other alternative pathways. In order to select the best combination to engineer, pathways were ranked according to several cost factors[18] as mentioned here. The best gene to be inserted into the chassis host *E. coli* according to the predicted result is 'lch: Lcho_0641' (Table 2) which is found in the KEGG database. This gene is an enzyme called nucleotide sugar dehydrogenase from *L. cholodnii*. The result indicates the ranked list of best pathways which also contains the number of genes involved, reactions, reaction cost, and additionally the best gene to be inserted into the chassis *E. coli* (Table 2). The overall cost of pathway insertion according to this study is 0.72. This is an individual sum of the reaction cost and gene cost (Table 2).

FBA was performed to evaluate the performance of *in silico E. coli* metabolic engineering capabilities for 1-butanol production. The methods of FBA and pFBA were applied using the OptFlux ver. 3.06 software platform on the genome-scale metabolic network of *E. coli* metabolism *i*AF1260[19] to predict addition and/or overexpression of nucleotide sugar dehydrogenase (*nsdh/b3544*) coupled with gene knockout strategies for producing 1-butanol in *E. coli*. The network model includes 1260 metabolic enzyme-coding genes accounting for 2382 reactions and 1668 metabolites. Focusing on glucose minimal medium, we applied the method to 1-butanol that can be secreted from *E. coli* after addition of one metabolite and two reactions (butanol and eq. (1)). Fixed OUR of 5 mmol g DW^{-1} h^{-1} was selected for this study as described in materials and methods because oxygen concentration has been experimentally reported to affect 1-butanol production in *E. coli*[5].

A preliminary sensitivity analysis was evaluated on the mutant model carrying *nsdh/b3544* gene, where the secretion profiles of acetate, formate and ethanol were maintained (Figure 3) as the wild type model developed elsewhere[19]. The mutant model maintained its viability and growth rate on glucose minimal medium as the wild-type (Figure 4). The fact that the mutant model guaranteed production rates of metabolites under glucose

minimal medium as the wild type makes our approach a promising start to *in vivo* metabolic engineering for 1-butanol production in *E. coli*.

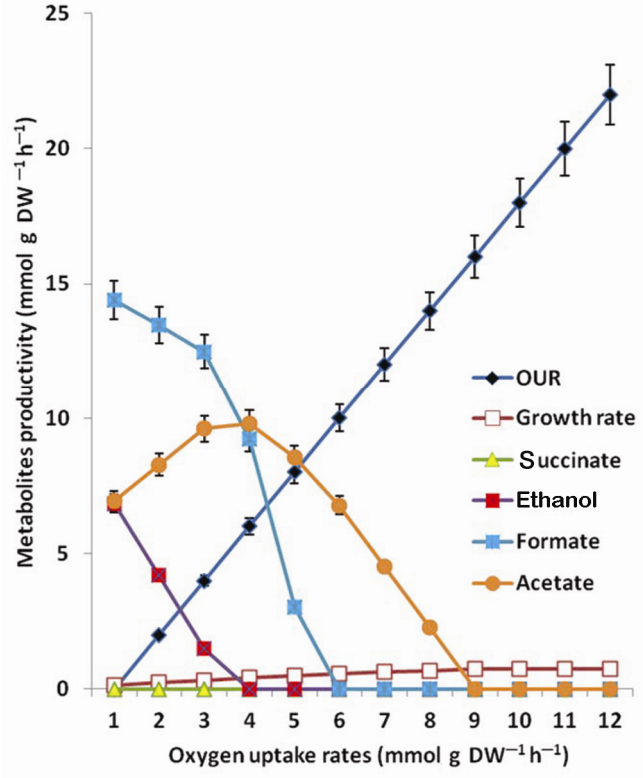

Figure 4. Metabolite secretion profile of the mutant model. All simulations were conducted with fixed glucose uptake rate of 8 mmol g DW^{-1} h^{-1} while varying oxygen uptake rate (OUR). Results represent the means of three determinations. Error bars indicate variation from the mean that is not more than 5%.

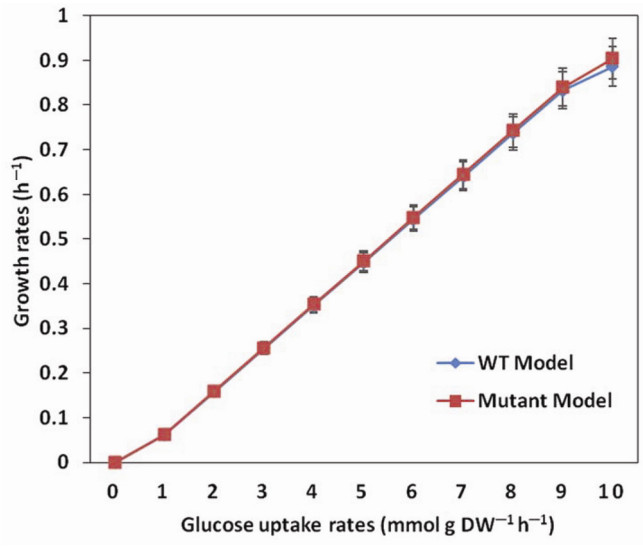

Figure 5. Glucose growth profile of the models: Wild-type (WT) model[19]; Mutant model (WT model + *nsdh* gene). All simulations were performed at fixed oxygen uptake rate of 18.5 mmol g DW^{-1} h^{-1} while varying the glucose uptake rates. Results represent the means of three determinations. Error bars indicate variation from the mean that is not more than 5%.

In silico mixed acid fermentation reactions that consume NADH were deleted (Figure 5) to achieve high titre 1-butanol in *E. coli* as reported elsewhere[4,5]. The mutant model growth under aerobic, semi-anaerobic and anaerobic conditions was evaluated (Figure 5). As expected, mutant model BSM1014 fails to grow under anaerobic condition. This is because it was previously reported experimentally that NADH-consuming pathways play a central role in *E. coli* anaerobic growth, as such the knockout of NADH-consuming metabolic pathways lead to lack of electron sink under anaerobic conditions[4].

nsdh/b3544 in silico overexpression under the OptFlux software platform using a simulation method of pFBA as described previously[25] was also evaluated on the two *E. coli* mutant models (BSM101 and BSM102) (Table 1). Considering the effect of oxygen concentration on 1-butanol production in *E. coli*, we performed the experiment with a constrained oxygen uptake rate of 5.0 mmol g DW^{-1} h^{-1} (ref. 5). Butanol reaction flux values of 5.4377 mmol g DW^{-1} h^{-1} for BSM101 and 5.5921 mmol g DW^{-1} h^{-1} for BSM102 were achieved after overexpression of the predicted gene (Figure 3). Significant increase in butanol flux (2.8%) was achieved in BSM102 mutant model, this might be a result of the deletion of NADH-consuming pathway genes.

We demonstrate *in silico* prediction of metabolic engineering strategies for 1-butanol production in a *E. coli* model reconstructed by Feist *et al.*[19], to combine pathway predictions using RetroPath with the addition of predicted metabolic pathway gene/reaction into the *E. coli* stoichiometric model using the OptFlux software platform (OptFlux ver. 3.06). In contrast to the previously reported experimental trial and error method for 1-butanol production in *E. coli* using traditional butanol fermentative pathways from *C. acetobutylocum*[4,5,9], here we present a novel *de novo* predicted pathway reaction/gene from *L. cholodnii* that has been shown to catalyse *in silico* 1-butanol biosynthesis in *E. coli.*

nsdh/b3544 that uses NADH and NADPH in the presence of butanal to produce butanol (eq. (1)) forms part of butanoate metabolism. This enzyme functions in oxidoreductase activity, acting on the CH–OH group of donors, NAD or NADP as acceptor (eq. (1)). Microbial metabolism involving central oxidoreductase enzymes such as dehydrogenase and reductases usually has preferential binding specificity for one of these two major currency metabolites (NADH and NADPH)[10]. Their role in oxidative phosphorylation (NAD^+ to NADH conversion) and anabolic reactions ($NADP^+$ to NADPH conversion) define their metabolic functionality. We took the advantage of inserting the pathway gene (*nsdh*) into the *E. coli* to enhance the production of 1-butanol. Our RetroPath prediction results seem to be promising, because the individual sum of the reaction cost with the gene cost being designated as part of the overall pathway insertions costs is 0.72 (Table 1). The reaction cost is zero indicating that it

is derived from annotated data in the database and not from predicted data. The gene cost on the other hand, is slightly different from zero because the data was derived from prediction results. Pathways with less putative enzymes are always favoured and the result obtained here is zero putative (Table 1). This approach is unique, as it is the first attempt to use redox biochemical reaction to produce 1-butanol.

OptFlux software was used as a platform for *in silico* FBA and pFBA to predict metabolic engineering strategies for increased 1-butanol production using an original *E. coli* model constructed previously[19]. Reactions predicted to catalyse 1-butanol production in *E. coli* were added to the model (see materials and methods) and various environmental and genetic perturbations were evaluated. The mutant strain carrying the 1-butanol metabolite in eq. (1) retained its growth and metabolite secretion profile on glucose minimal media as did the wild-type model of Feist *et al.*[19] under the same environmental condition (Figure 5). The simulation results for preliminary estimation of maintenance parameters, particularly glucose growth and physiological predictions of growth rate and secretion profile, revealed consistency with the wild type model[19]. These results indicate that the mutant model retains its viability and secretion profile even after addition of 1-butanol (foreign metabolite), reactions and gene insertion (Figure 4).

In silico deletion of mixed acid fermentation reactions (succinate, lactate and ethanol) was evaluated on the mutant strains (Figure 3 and Table 1). Deletion of these NADH-consuming pathways have been established experimentally to increase the pool of NADH and acetyl-CoA which has a direct effects on high titre 1-butanol production in *E. coli*[4,5]. Our mutant strain survived *in silico* knockouts of *adhE/b1241* and *ldhA/b1380* under stated aerobic, semi-anaerobic and anaerobic environmental conditions (Figure 6) but only BSM1014 could not survive knocking out of all three (*b1280*, *b1241* and *b4152*) NADH-consuming pathway genes under anaerobic conditions (Figure 6). It also survived these deletions under aerobic and semi-anaerobic conditions. This model (BSM1014) lost its ability to grow anaerobically due to lack of its NADH-consuming pathways which act as an electron sink. The model is unable to recycle NADH, thereby creating a driving force for reactions that consume NADH[4]. This is a true positive simulation result because it was experimentally reported earlier[4]. Our simulation results using the models developed in this study sound promising, because they agree with experimental results reported elsewhere[4,5]. Given the results obtained herein, it could ultimately serve as a starting point for *in vivo* metabolic engineering implementation to increase 1-butanol production in *E. coli*.

A butanol reaction flux of 5.43771 mmol g DW^{-1} h^{-1} was achieved when *nsdh/b3544* was overexpressed using the pFBA under OptFlux software platform. On the other hand, the effect of NADH regeneration in relation to butanol production on the mutant strain was also evaluated, where knocking out NADH-consuming pathways genes showed a significant increase in butanol reaction flux from 5.4377 mmol g DW^{-1} h^{-1} to 5.5921 mmol g DW^{-1} h^{-1} (2.8%). We can hypothesize that as the *nsdh* expression level increases, the butanol flux also increases (Figure 3). This hypothesis is in agreement with the increase butanol production in *E. coli* reported previously by Atsumi[5,8]. Although, the scenario here is slightly different because the butanol fermentative pathway from *Clostridium* has not been used in this approach, instead a reaction predicted to catalyse 1-butanol production from *L. cholodnii* which was added into the *E. coli* stoichiometric model was used. It has been previously established that in *E. coli* central metabolism, the acetyl-CoA branch partitioning initiates NADH-consuming reactions[4]. As a result of this, we hereby propose a hypothetical-predicted reaction from *L. cholodnii* for 1-butanol production in *E. coli* using our novel *de novo* retro synthetic pathway prediction approach (Figure 1 *b*). In essence, the simulation of mutant strain with the three (3) gene knockouts[4,5] known to produce 1-butanol was carried out and a significant butanol flux rate was achieved as stated earlier. The mutant model was simulated constraining the oxygen uptake flux to 5 mmol g DW^{-1} h^{-1}. Limited oxygen uptake rate was selected for this study because it was experimentally reported elsewhere[5], that a slight increase in oxygen level increased the production of 1-butanol in *E. coli*. This

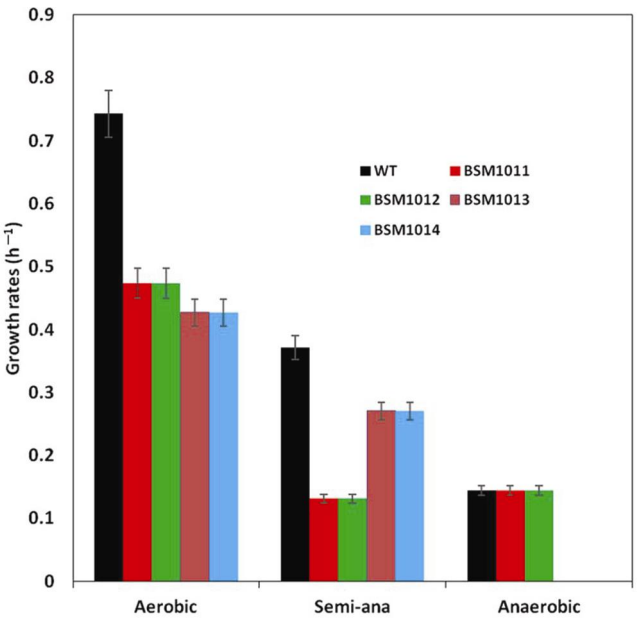

Figure 6. Growth profile of the mutant models with mixed acid fermentation genes knockout under aerobic, semi-anaerobic and anaerobic conditios. BSM1014 (Δ*adhE* Δ*ldhA* Δ*frdBC* + *nsdh* gene). WT model (Feist model + *nsdh* gene). Results represent the means of three determinations. The error bars indicate variation from the mean that is not more than 5%.

suggests that the NADH produced anaerobically might be insufficient to supply for the 1-butanol production. On the other hand, in a completely aerobic condition, *E. coli* consumes both acetyl-CoA and NADH in the tricarboxylic acid cycle and respiration, which ultimately contributes to decreased butanol production[5].

Finally, the notion for 1-butanol production in *E. coli* is not the first to bridge the world of metabolic and genetic engineering approaches[4,5,8,16,17], although it is the first to use a novel bio-retrosynthetic pathway gene in combination with an *E. coli* GSMM. In addition, this study evaluates the *in silico* addition of nucleotide sugar dehydrogenase (*nsdh/b3544*) using the OptFlux ver. 3.06, which catalyses 1-butanol biosynthesis in *E. coli.* A butanol reaction flux value of 5.43371 mmol g DW^{-1} h^{-1} has been demonstrated and the mutant strain is able to exhibit a substantial increase in butanol production by adding and overexpressing the predicted pathway gene (*nsdh/b3544*). The engineered *E. coli* (BSM102) was capable of increase in butanol production while maintaining the growth rate and metabolite secretion profile as the wild-type. Although we do not know the exact mechanisms involved in coupling the bio-retrosynthetic pathway reaction/gene with *E. coli* central metabolism, we proposed a hypothetically predicted reaction leading to 1-butanol production in *E. coli*. *E. coli* has been extensively explored as a chassis host for butanol production, but butanol toxicity is a major concern. Recent successes have been reported in engineering of *E. coli* strains for butanol tolerance; ranging from the use of artificial transcription libraries[26] and compound toxicity prediction[27,28]. In addition, *n*-butanol tolerance mechanisms conferred by membrane-related genes have been reported elsewhere[29], while membrane targeted tilapia metallothionein was reported to have improved *n*-butanol tolerance in *E. coli*[30]. Recently, global analyses of *E. coli* stress responses under various exogenous biofuels, including *n*-butanol were conducted and extensively reviewed previously[31]. Indeed, an exciting prospect for future studies will bridge our findings with *in vivo* 1-butanol production in *E. coli* using retrosynthetic biology approach.

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Morphometric analysis of Barren volcanic base and associated tectonic elements in the Andaman fore-arc sub-basin

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Although many studies have been undertaken on the Barren Island, the undersea configuration of the Barren Island Volcano (BIV) and associated morphotectonic elements, the modification imparted on them by the recent flows and the morphology of these flows are still poorly known. The present study identified thick lava flows along the western and southwestern slopes of Barren Island, extending up to the base of the Invisible Bank. The feeding locales for these thick flows are numerous parasitic cones picked up in the 3D grid model generated by the multibeam echo sounder. Morphology of the Barren volcanic base is largely modified by recent eruptions as they not only reduced the steepness of the Barren Volcano along the western slope, but also extended the base of the Barren Volcano to the base of the Invisible Bank. Furthermore, the present study also brought out other morphotectonic elements surrounding the BIV, viz. Alcock seamount towards the southeast, Invisible Bank flanked by west Andaman Fault in the west and a newly identified submarine volcanic mount as part of the Andaman Volcanic Arc towards the south.

Keywords: Alcock seamount, barren volcano, geomorphology, west Andaman Fault.

BARREN Island Volcano (BIV) is India's only active volcano lying about 135 km ENE of Port Blair within the exclusive economic zone (EEZ) of India. After 150 years of quiescence, it became active for five years from 1991 to 1995. BIV became active once again after a gap of 12 years on 28 May 2005. Being a part of the Neogene Inner Volcanic Arc (NIVA), BIV is located in the Andaman Sea which is a marginal sea on the western side of the Burmese continental mass marked with an oblique back-arc spreading centre[1,2]. Pal *et al.*[3] described the geology of the BIV with excellent descriptions, analyses and interpretations. This belt can be traced from the Central Molasse basin of Myanmar, through the Narcondam and BI into the structural trend of Sumangko rift zone, along the volcanic Barisan range axis of northern Sumatra[4] (Figure 1). Other important tectonic elements in the Andaman Sea are the Invisible Bank (IB), Diligent Fault (DF) and West Andaman Fault (WAF). IB is formed by thick lava flows overlain by sedimentary rocks of Middle Miocene extending to the east of Andaman and Nicobar Group of Islands, whereas DF and WAF form the western and eastern margin towards the northern part of the Andaman Sea respectively[2,5].

No attempts have been made so far to understand the undersea configuration of the BIV and associated morphotectonic elements. Furthermore, understanding of the extent of recent flows and their morphology is of paramount importance for understanding the evolution of the BIV. In addition, the disposition of other morphotectonic elements around the BIV is not yet understood in detail. With these objectives in mind, a full-fledged cruise SM-220 was mounted on-board *RV Samudra Manthan* to obtain the 3D sea-bed morphology of the Barren volcanic cone and associated geomorphic–tectonic features in the Andaman Sea. To bring out the undersea configuration of the Barren Island (BI) and surrounding morpho-tectonic elements, multibeam swath bathymetric survey was carried out in an area 5080 sq. km around Barren Island with

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