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# From In Silico Prediction To In Vivo Validation: Advancing Biohydrogen Production Through Metabolic Engineering Of E. Coli MG1655

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***Abstract***
*Metabolic engineering and synthetic biology advanced in recent times resulting in plenty of methods and tools for genetic manipulation rationally. This study explains the application of Genome-scale metabolic model and its experimental validation using biohydrogen production as a case study. The iJO1366 GEM was explored to identify the probable targets for the metabolic engineering Escherichia coli MG1655 strain by simulating its anaerobic growth in three different carbon sources demonstrating its versatility to predict accurate phenotypes. Moreover, availability of advanced engineering tools such as CRISPR has aided the field of metabolic engineering increasing the efficiency of genetic intervention. Therefore, this work also demonstrates the application of CRISPR-Cas9 technology and its modification to ease it's the process of screening out successful mutants. Developed methodology follows primer based mutant screening and avoiding the need of sequencing process which saves time and resources. Utilizing this knowledge, this study uses E. coli as a chassis to illustrate ΔhybC mutant construction and its application for biohydrogen production using different carbon substrates emphasising the value of substrate based on the growth phase of the cell.*
***Keywords****: E. coli, iJO1366, biohydrogen, glucose, CRISPR*

## 1. INTRODUCTION

Metabolic engineering is an integrated part of today's industrial biotechnology and has been applied to vast number of bioprocesses aimed for the mass production of chemicals, enzymes, food additives, biofuels, etc [1]. The metabolic engineering brings the directionality in the field of genetic engineering making it a rational process of choosing genetic changes [2]. This has been proved a powerful technology that helps to address changes such as knockout, knockdown, overexpression, pathway engineering to obtain certain targeted specific compound [1]. Complementary to this, advancements in new and advanced methods and tools developed for DNA modification have aided the metabolic engineering. Techniques such as Biobrick assembly, Lambda-Red recombinase-based engineering also known as recombineering, MAGE, and tools such as zinc-finger nucleases (ZFNs), TALENS (transcription-activator like effector nucleases), etc. have elevated the field of metabolic engineering [3, 4]. These modern tools provide specificity, accuracy, and are efficient to modify the genome with minimal or no error [3]. Among which CRISPR-Cas (Clustered regularly interspaced palindromic repeats/ CRISPR associated protein) has revolutionised the field of metabolic engineering as it offers high efficiency with low-cost operation [5]. The CRISPR-Cas system is dynamic and adopted from immune system of bacteria which is RNA-guided system [6]. This system is a powerful tool as it can recognize target sequence with a very short sequence of RNA, 20 bp, and delivers the specificity better than other tools developed before [7]. CRISPR-Cas system containing Cas-9 protein was repurposed with small guide RNA (sgRNA) resulting into two-component system which facilitated the editing experience in host cells such as E. coli.

Parallel to these advancements the advents in the "-omics" era together with synthetic biology and metabolic engineering established the endeavours of synthetic biology. Synthetic biology and its contribution towards metabolic engineering have paved a way to reconstruct biological connections mathematically resulting in systems biology. The genome scale metabolic models (GEMs) are curated based on the knowledge available for gene-protein reactions of an organism [8]. These models allow users to identify a gene or a reaction and its association with the metabolic pathways empowering its application to predict a metabolic phenotype mathematically instead of experimental trial-and-error method, delivering accurate predictions. There are various GEMs available for model organisms, such as for Escherichia coli, Saccharomyces cerevisiae, etc, these reconstructions of genome-scale networks aid in simulating a GEM to predict an outcome based on constraint-based modelling which delivers closest prediction of metabolic phenotype of an organism based on the method (FBA, MOMA, ROOM, etc).

GEMs have been proved as powerful tools in the field of metabolic engineering and are reported to have aided number of studies to achieve desired target phenotype [9]. Similarly, in response to the concern towards declining fossil fuel reserves and climate change, there is an urge to switch to a sustainable fuel in future for energy requirements. Biohydrogen is an ideal biofuel for future applications and its biological production using microbial cells makes it a sustainable option as a renewable resource.

Hydrogen is an energy dense molecule with 141 MJ/kg gravimetric energy density which is highest. Unlike other conventional fossil fuels, hydrogen combustion results only in water as a byproduct proving to be the cleanest burning fuel. The conventional industrial production of hydrogen is an energy-intensive process requiring fossil fuels as a raw material. The hydrogen produced from different types of raw materials has rendered it to be categorized in various classes which are color coded depending on the raw material used for the production [10, 11]. Biohydrogen production via dark fermentation is a most sought process as it involves microbial cells as a chassis and waste renewable resources as a raw material [12, 13]. Biohydrogen production via E. coli using waste resources such as waste paper pulp, brewery spent grains, etc have been reported [14, 15]. Whereas, lignocellulosic waste is an ideal raw material in terms of abundance and availability which is used as a low-cost feedstock for the dark fermentation for biohydrogen production [16]. Moreover, to achieve the scalable amounts of biohydrogen produced via this method will require enhancing the productivity in E. coli to meet the future demands. Experimentally performing such elaborated changes to E. coli genome to optimise its biochemical native Pyruvate formate lyase (PFL) pathway is a tedious and labour-intensive process [17]. To make this process cost-effective, many studies have sought the application of GEMs to identify targets to enhance hydrogen production in E. coli [18, 19]. In silico analysis for predicting a metabolic phenotype to enhance the hydrogen productivity using low-cost substrate is a strategically demanding and sustainably suitable option. This method preserves the resources and time to achieve desirable results in metabolic engineering. Here we demonstrate a reliable target selection and illustrate knockout of a gene using CRISPR-Cas9 based methodology of systematically modifying the E. coli chassis representing hydrogen production as a case study.

## 2. MATERIALS AND METHODS

### In-silico simulation conditions

In-silico simulations were performed using iJO1366 GEM of E. coil MG1655 on OptFlux platform [8, 20, 21]. Knockout of gene was performed by constraining the lower bound to ‘0’, where ‘1’ means WT expression of gene. The anaerobic condition was simulated by constraining the model to have lower bound of oxygen exchange to ‘0’. Whereas, the carbon substrate used was constrained to have lower bound of -10 mmol/gDW/h at a time as only one substrate was used at a time and others were kept zero to reflect single substrate utilization.

### Microbial strains, plasmids and culture conditions

Wildtype E. coli MG1655 K-12 (K-12 F– λ– ilvG– rfb-50 rph-1) strain was received from kind donation of Dr. Vijai, Indrasheel University, Gujarat, India, and was used for biohydrogen production. Overnight grown primary culture (OD 0.8) was used for inoculating secondary culture with 2% v/v for growth curve and biohydrogen production. Anaerobic fermentation for biohydrogen production was performed with 50 mL media in serum bottles (125 mL) with butyl rubber stopper sealed by aluminium crimps and sparged with nitrogen gas for 15 minutes. Cultures were incubated at 30 °C at 180 rpm on benchtop shaker incubator (LabWit Benchtop Shaking Incubator, ZWY-240, Shanghai, China) and were withdrawn at 24 Hr for hydrogen quantification.

Minimal M9 media (1X) was used and prepared using 5X stock (HiMedia Cat. No: G013-500G), supplemented with yeast extract (HiMedia Cat no.: RM027-500G), $MgSO_4$ (HiMedia Cat no.: TC146-500G), carbon substrate and $CaCl_2$ (Finar Cat no.: 10430SG500). Carbon substrates used were glycerol (20% aq.; 2 mL), glucose, arabinose and xylose, where lignocellulosic mixed sugars are represented as a synthetic composition of glucose (4.8g/L), xylose (2.8g/L) and arabinose (0.4g/L) to mimic fermentable lignocellulosic hydrolysate. Fermentation control comprised of only glucose (8 g/L) as a carbon substrate. Mutant selection involved antibiotics ampicillin (100 mg/L), kanamycin (50 mg/L) and spectinomycin (50 mg/L) and for induction IPTG (0.5M) was used and added as required. Plasmids and primers used in this study are given in Table 1.

**Analytical methods**

Hydrogen gas was quantified using gas chromatograph equipped with thermal conductivity detector (Thermo Scientific TRACE 1610, Italy). The sample gas (500 µL) was injected with gas-tight syringe and argon gas was used as a carrier gas. Chromatograms were acquired and analysed using Chromeleon-7 software. Growth of the culture was analysed using Biotek Cytation-5 multimode reader (USA), equipped with Gen-5 software.

Table 1. List of plasmids and primers used in this study.

| Name | Specifications | Reference |
|---|---|---|
| pCas | $Kan^r$ | Addgene ID #62225 |
| pTargetF | $Spec^r$ | Addgene ID #62226 |
| pUC18 | $Amp^r$ | Addgene ID #50004 |
| hybC-TarF-F | ACGGCAAAACGGTAGAAGTGGTTTTAGAGCTAGAAATAGCAAGT | |
| hybC-TarF-R | CTTCTACCGTTTTGCCGTACTAGTATTATACCTAGGACTGAGCT | |
| R2-Up-hybC-F | CAGGAAACAGCTATGACGGAATGTTCGGCTTCACAAA | |
| R2-Up-hybC-R | TTATTATTATTATTATTACTGAATGAAATCGTTGCGAT | |
| R3-Dwn-hybC-F | TAATAATAATAATAATAAGAATGGTGGTGCCTTCCCAC | |
| R3-Dwn-hybC-R | GTTTTCCCAGTCACGACCGTGCCGGAATTCCCGACCG | |

## 3. RESULTS AND DISCUSSION

**Gene target scrutiny under glucose, glycerol and xylose**

In-silico analysis of single gene deletion across three different carbon substrates were performed using iJO1366 genome scale model to predict the gene targets that enhances the formate production. Formate produced in cell is further converted into hydrogen gas by pyruvate formate lyase (PFL) pathway and therefore being a penultimate carbon-containing compound of the PFL pathway, formate is traced in C13 labelling while constructing a GEM and is proportional to the hydrogen produced. In this work, three sugar substrates are employed, glycerol, xylose and glucose, among which glycerol is an industrial by-product and other two are part of lignocellulosic waste biomass.

Cellular biomass was studied when these three sugar substrates were used individually and anaerobically. It is necessary to maintain a minimal cell biomass after gene perturbation in order to survive and produce the targeted metabolite. When E. coli is grown anaerobically, hetero-fermentation takes place resulting in production of mixture of carboxylic acids (acetate, succinate and lactate) and alcohol (ethanol). To increase the flux towards PFL pathway we demonstrated the in-silico knockout of genes that scavenge the carbon-flux from PFL pathway. Seven genes (ldhA, adhE, hyaB, hybC, hyfG, focA and frdA) were knocked out and compared for their behaviour in glucose, xylose and glycerol to compare the suitability towards

formate production.

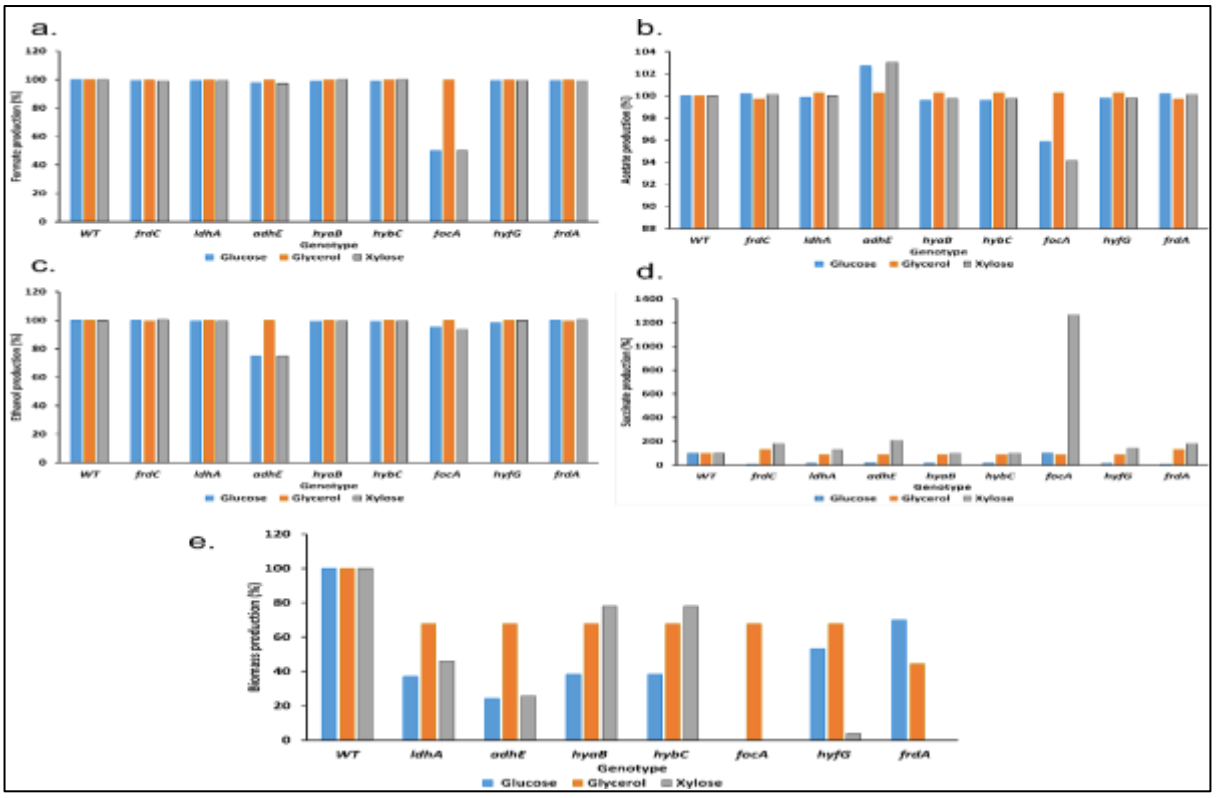


*Figure 1. In-silico analysis of gene knockouts in iJO1366 GEM of E. coli MG1655 in anaerobic conditions. a. Formate production; b.Acetate production; c. Ethanol production; d. Succinate production; e. Biomass production*

When grown in glycerol, the cell biomass declined to 44.43% in ΔfrdA mutant responsible for succinate production which competes for carbon flux and was lowest among all the mutants studied (Fig 1). Whereas, mutants when grown in glucose, ΔfrdA mutant resulted in highest biomass production reaching to 70.06% which was peculiar when compared across other sugar substrates highlighting the effect of different substrates on the biomass. While glucose being a primary sugar substrate for E. coli, no growth was observed in ΔfocA mutant stating its essentiality for cellular growth, and only 24.27% biomass was observed in ΔadhE mutant, which was lowest in glucose. The lowest biomass reported in ΔadhE mutant responsible for ethanol production implying the importance of this gene for cell survival as it catalyses the reaction which regenerates the redox species in cell to fuel the next cycle of glycolysis. When these mutants were grown in xylose as a substrate, ΔfocA andΔfrdA mutants resulted in no growth implying their essentiality for survival of the cell and therefore could not be knocked out. Whereas, the lowest biomass was observed in the ΔhyfG mutant resulting in 3.71% biomass production. Across all the sugar substrates studied, mutants exhibited repressed biomass growth compared to WT implying the effect of altered metabolism after genetic perturbation of cell.

The formate production in these mutants across all the sugar substrates resulted in equivalent formate production when compared to the respective wildtypes except few instances shown in Fig 1. Formate production drastically declined upto 50% (when grown in glucose or xylose as a substrate) when formate transporter focA was knocked out stating its importance in maintaining cellular viability as discussed earlier and depicted in Fig 1. Also formate over production leads to intracellular toxicity and therefore, focA acts as a channel that transports formate to extracellular space preventing the cellular death and maintaining the redox potential across the cellular membranes. Every other mutant studied in in-silico did not hamper formate productivity except ΔfocA mutant.

Apart from PFL pathway, the pyruvate flux from central metabolic system is fed towards acetyl-CoA production which is co-produced as a result of pflB catalyses splitting pyruvate into formate and acetyl-CoA. The conversion of pyruvate into acetyl-CoA and is followed by production of ethanol and acetate. The reaction of these products formation is coupled with the regeneration of reducing equivalents

produced as a result of glycolysis which helps in continuing of cycle. Therefore, ethanol and acetate production is indispensable and result as a co-fermentative product of hydrogen production also reported previously. Ethanol production was declined to ~75% when grown in glucose and xylose individually in ΔadhE mutant, as protein of this gene catalyses the reaction of acetaldehyde to ethanol conversion in E. coli. In contrast, no change in ethanol production was seen when ΔadhE mutant was grown in glycerol. Whereas, acetate production in all mutants across all the condition was significantly unaltered when compared to WT.

This experiment demonstrates the application of iJO1366 GEM to predict the metabolic phenotype of E. coli which would otherwise take days to analyse if performed experimentally, saving time and resources. Further this prediction was validated experimentally usingΔhybC mutant to demonstrate the hydrogen production. The hybC mutant was constructed using CRISPR-Cas9 technology with modifications to strategy reported by Jiang et.al [22].

**Construction of clone: modified CRISPR methodology for constructing and screening of mutants**

CRISPR-Cas9 technology and its advancements has revolutionized the field of metabolic engineering [23]. Cloning strategy developed to construct mutants of targeted genes was adopted from Jiang et. al., which has developed a two-plasmid system using recombineering technology [22]. This technology enables to use specificity of CRISPR system aided by λ-Red system to continually edit genome of E. coli. This technology has been proved to be effective in genome editing of systems other than E. coli insisting on its diverse applications [22]. We have adopted this two-plasmid system for metabolic engineering of E. coli to enhance the metabolic flux towards formate. To integrate the $N_{20}$ sequence in pTargetF, which was incorporated using overlap-extension PCR method and was not restriction-digestion based as reported (Fig. 2a.). The primers designed consisted an overhang of targeted $N_{20}$ region at 5'-end of primer with 3' complementary region to sgRNA scaffold of the pTargetF. Amplification was done using pTargetF as a template using primers as explained which resulted in added $N_{20}$ sequence between the sgRNA scaffold. Clones were confirmed by sequencing.

Donor dsDNA or donor brick were used to repair double strand breaks induced by CRISPR-Cas system in the E. coli genome by homologous recombination (Fig. 2b.). Repair was mediated through λ-Red recombinase system by recombining the dsDNA donor with the E. coli genome. The second change made was to ease this step of clone selection, we modified the selection strategy by designing the primers which differentiates between WT and mutant and completely omits the necessity of confirmation by sequencing. The original strategy involved the mutant selection by sequencing which is tedious, time and resource consuming. Therefore, to circumvent this, the donor brick was designed to have homologous sequence of 200bp from upstream and downstream of $N_{20}$ protospacer separated by six stop codons comprising of 418 bp. To facilitate the repeated use of donor brick it was cloned into the pUC18 vector's MCS.

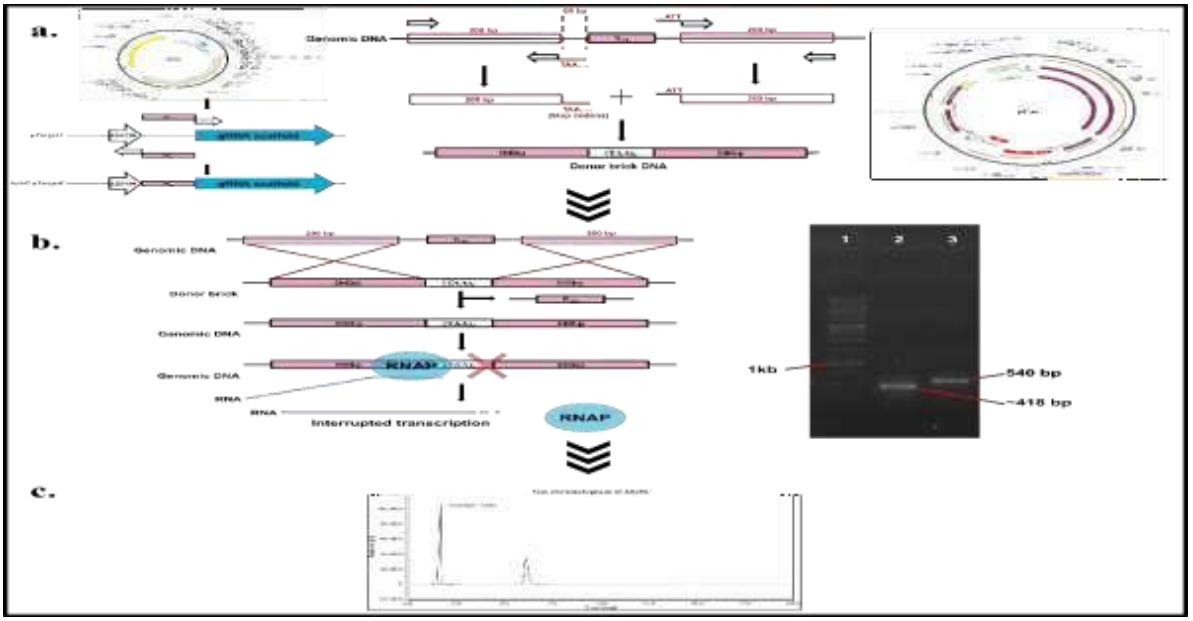

Figure 2. Methodology of CRISPR-Cas9 based two-plasmid system for experimental knockout in E. coli MG1655.

We amplified 200bp regions of upstream and downstream regions 60 bp away from target $N_{20}$ protospacer in E. coli genome around each target site (Fig. 2a.). To do so, we used separate sets of primers for the amplification of upstream and downstream segments. For upstream homologous sequence primers were designed to have an overhang of 17 bases at the 5'-end of forward primer, which was complementary to the sequence at MCS termed as Region-2' or simply as R2' with sequence 5'-caggaaacagctatgac-3'. And the reverse primer for upstream sequence has six stop codons at 5'-end of primer to induce transcriptional arrest if any RNA is transcribed will be truncated. This complementary stop codon overhang is also present at 5'-end of forward primer for downstream homologous sequence so that the donor brick comprises of homologous regions separated by 6 stop codons. Whereas, reverse primer for downstream sequence was attached with the overhang of 17 bases complementary to the Region-3 (R3) of pUC18 with sequence 5'-GTTTTCCCAGTCACGAC-3'. Thus, a donor brick overall has a size of 418 bp as shown in Figure 2a. Using these constructs knockout was performed to obtain ΔhybC mutant to demonstrate the hydrogen production in E. coli MG1655 (Fig. 2b).

**Gene editing of hybC in E. coli MG1655**

Based on our in-silico analysis, we demonstrate gene knockout experiment of hybC in E. coli MG1655 (Fig. 3). The hybC-pTargetF and donor brick were cloned as explained above. Single-gene knockout was performed in E. coli MG1655 competent cells containing pCas and induced by λ-Red recombinase (10mM arabinose). Competent cells were co-transformed with the hybC-pTargetF and donor brick initially using recommended concentration of 100 ng and 400 ng, respectively. The transformants were incubated at 30ºC overnight which yielded in no positive mutants. The optimization strategy involved the use of increased DNA concentrations of pTargetF (up to 200 ng) and donor brick (up to 600 ng) to increase the probability of achieving target deletion, which yielded in 28.5% of knockout efficiency. Colonies obtained were screened for knockouts using upstream forward and downstream reverse primers, which resulted in 540 bp of WT amplification and a mutant of 418 bp as depicted in gel picture shown in Fig. 2b.). The mutants were then isolated and subjected for growth and hydrogen production studies.

**Hydrogen production in E. coli: analysing substrate compatibility**

To validate the in-silico analysis and the modified CRISPR-Cas9 system constructed, we performed knockout of hybC gene by avoiding the need of removing the whole coding gene and instead only removed a stretch of 140 bp to abrupt WT gene expression hindering its normal functioning (Fig. 3). The carbon substrate suitability to grow this mutant and produce hydrogen was analysed. The anaerobic growth of the WT and ΔhybC mutant was analysed in glycerol and it was found that the glycerol as a sole carbon substrate did not support the growth or hydrogen production (Fig. 4). The anaerobic growth curve derived, depicted the negligible growth, unable to sustain hydrogen production (0.9%) (Fig. 5). As cell growth is an import

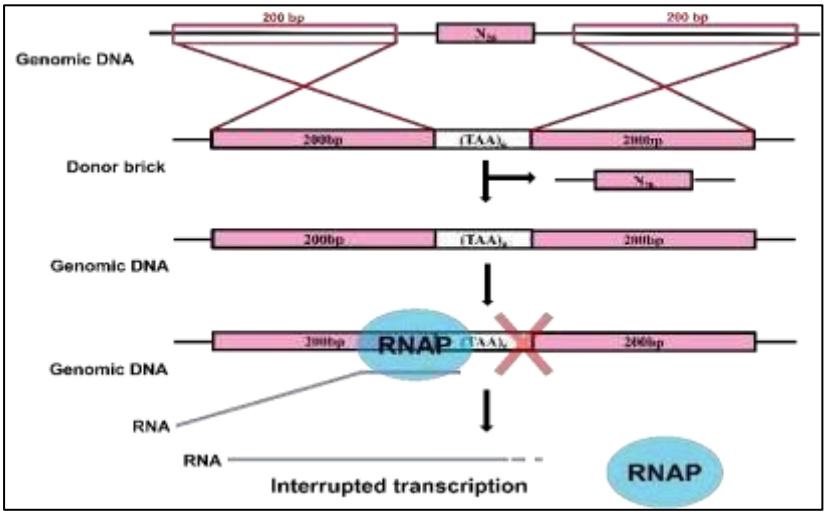


*Figure 3. Diagram depicting gene disruption strategy using pCas and pTargetF.*

ant factor to obtain maximum metabolite production and it is evident from many studies, cell vitality plays an important role. The overall growth can be divided into two phases of growth and production of hydrogen where in former stage cell growth is prioritize and fed with a carbon substrate that supports the cellular growth [24]. From our previous observations, and Fig 1, it is evident that growth was supported by glucose and xylose as a substrate sugars. Therefore, the cells were first cultivated in glucose or mixed sugars and then glycerol was added as substrate after 6 hr of growth for hydrogen production. There are similar studies that support this two-stage process of target metabolite production where fermentation is divided into two distinct stages of biomass growth and production proving an effective strategy for fermentative production of metabolites [24]. Hydrogen production inΔhybC mutant when grown initially in glucose and later glycerol addition resulted in 21.58± 1.8 % hydrogen which was highest (Fig 5). This implies the better utilization of glycerol in the production stage of hydrogen as the cell biomass reaches appropriate volume. Contrarily, negligible hydrogen was produced when the mutant was grown in medium with only glycerol as a substrate in both stages supporting the observed insignificant growth in

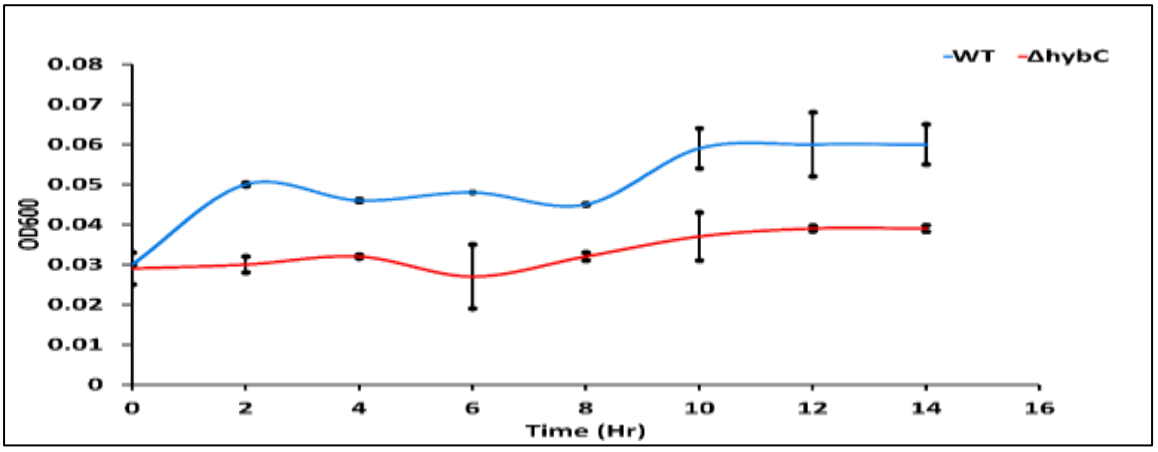


Fig 4.

*Figure 4. Anaerobic growth curve of E. coli MG1655 wildtype (WT) and ΔhybC mutant in glycerol as a carbon substrate.*

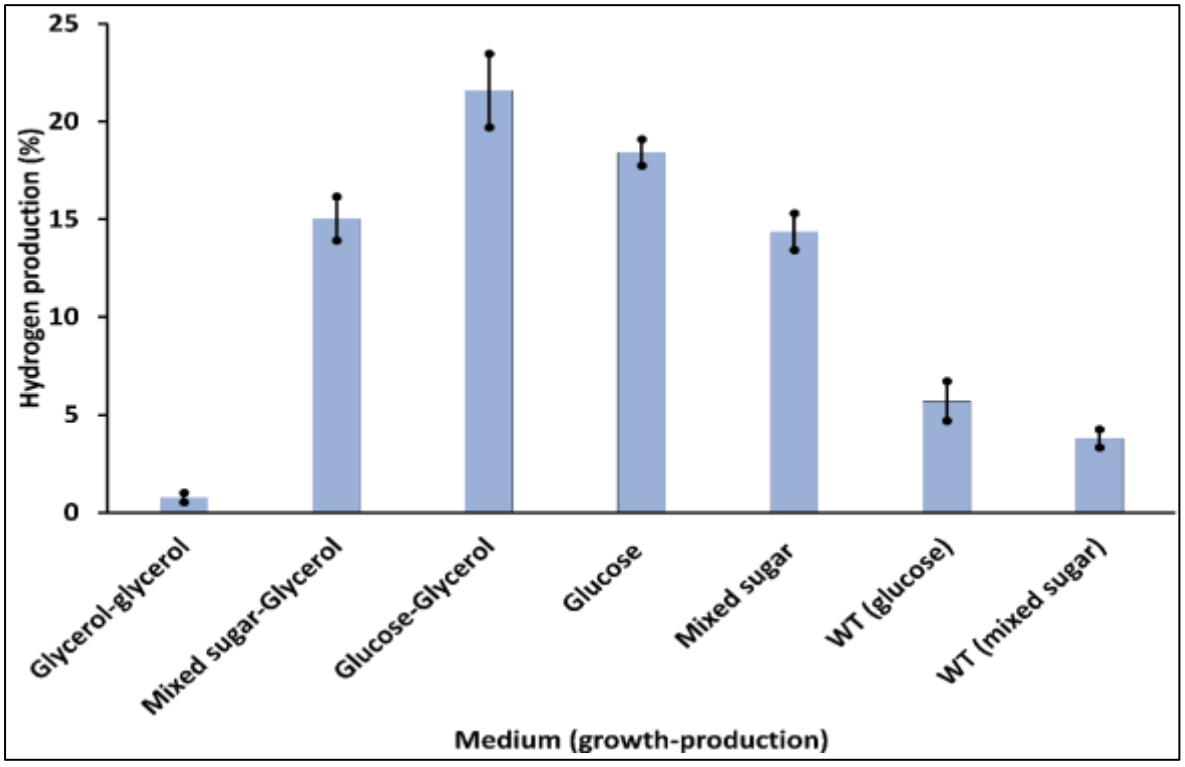


Figure 5. Hydrogen production in anaerobic conditions with different carbon substrates.

Advancements in GEMs and their reliability to predict the metabolic phenotype after simulating genetic perturbations have aided the system and metabolic engineering field. Utilizing this knowledge we have demonstrated a case study of biohydrogen production utilizing various sugar substrates. Also, the genomic perturbation in E. coli has been revolutionized since the discovery of the CRISPR-Cas9, an inherent anti-viral system repurposed for the precise editing of bacterial genome. This study advocates for the application of advancements in the systems and synthetic biology as illustrated here with the help of the iJO1366, a GEM which could successfully predict the metabolic phenotype using OptFlux, a user-friendly tool.

## 4. CONCLUSION

Metabolic engineering of a microbial cell is a tedious task and therefore advancements in synthetic and systems biology have aided the process of genetic manipulation. The GEM iJO1366 has been used widely to simulate the phenotypic changes in E. coli after genetic perturbations and helped to predict an accurate result. Similarly, we have demonstrated an application of this GEM to identify the gene targets and how to apply this knowledge for experimental setups. Also, CRISPR technology was used in this study was modified to make the mutant selection easier which bypasses the need of sequencing to confirm the knockout. Third, we have demonstrated the utilization of glycerol for hydrogen production by adding it in later stages of growth as glycerol as a sole carbon source was unable to support cell biomass generation.

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**REFERENCES**:

1. Y.-S. Ko, J.W. Kim, J.A. Lee, T. Han, G.B. Kim, J.E. Park, and S.Y. Lee, Tools and strategies of systems metabolic engineering for the development of microbial cell factories for chemical production, Chemical Society Reviews. 49(14) (2020) 4615-4636, 10.1039/D0CS00155D.
2. G. Stephanopoulos, A.A. Aristidou, and J. Nielsen, Metabolic engineering: principles and methodologies, (1998).
3. K.R. Choi, W.D. Jang, D. Yang, J.S. Cho, D. Park, and S.Y. Lee, Systems metabolic engineering strategies: integrating systems and synthetic biology with metabolic engineering, Trends in biotechnology. 37(8) (2019) 817-837.
4. R.P. Shetty, D. Endy, and T.F. Knight Jr, Engineering BioBrick vectors from BioBrick parts, Journal of biological engineering. 2(1) (2008) 5.
5. D. Zhao, X. Zhu, H. Zhou, N. Sun, T. Wang, C. Bi, and X. Zhang, CRISPR-based metabolic pathway engineering, Metabolic Engineering. 63(2021) 148-159.
6. P. Horvath and R. Barrangou, CRISPR/Cas, the immune system of bacteria and archaea, Science. 327(5962) (2010) 167-170.
7. W. Jiang, D. Bikard, D. Cox, F. Zhang, and L.A. Marraffini, RNA-guided editing of bacterial genomes using CRISPR-Cas systems, Nature biotechnology. 31(3) (2013) 233-239.
8. J.D. Orth, T.M. Conrad, J. Na, J.A. Lerman, H. Nam, A.M. Feist, and B.Ø. Palsson, A comprehensive genome-scale reconstruction of Escherichia coli metabolism—2011, Molecular systems biology. 7(1) (2011) 535.
9. S.J. Lee, D.-Y. Lee, T.Y. Kim, B.H. Kim, J. Lee, and S.Y. Lee, Metabolic engineering of Escherichia coli for enhanced production of succinic acid, based on genome comparison and in silico gene knockout simulation, Applied and environmental microbiology. 71(12) (2005) 7880-7887.
10. V.J. Aimikhe and O. Eyankware, Recent advances in white hydrogen exploration and production: a mini review, Journal of energy research and reviews. 13(4) (2023) 64-79.
11. J.M.M. Arcos and D.M. Santos, The hydrogen color spectrum: techno-economic analysis of the available technologies for hydrogen production, Gases. 3(1) (2023) 25-46.
12. T.B. Madavi, S. Chauhan, M. Jha, K.-Y. Choi, and S.D.V.N. Pamidimarri, Biohydrogen Machinery: Recent Insights, Genetic Fabrication, and Future Prospects, Chemical Engineering & Technology. 46(2) (2023) 179-190, https://doi.org/10.1002/ceat.202000527.
13. T.B. Madavi, S. Hariharno, A. Singh, S. Chauhan, and S.D. Pamidimarri, Biofuels: Fueling the Future with Whole-Cell-Derived Fuels, in Whole-Cell Biocatalysis. 2024, Apple Academic Press. p. 309-334.
14. A. Poladyan, K. Trchounian, A. Vassilian, and A. Trchounian, Hydrogen production by Escherichia coli using brewery waste: optimal pretreatment of waste and role of different hydrogenases, Renewable Energy. 115(2018) 931-936.
15. A. Poladyan, L. Margaryan, K. Trchounian, and A. Trchounian, Biomass and biohydrogen production during dark fermentation of Escherichia coli using office paper waste and cardboard, International Journal of Hydrogen Energy. 45(1) (2020) 286-293.
16. P. Sivagurunathan, G. Kumar, A. Mudhoo, E.R. Rene, G.D. Saratale, T. Kobayashi, K. Xu, S.-H. Kim, and D.-H. Kim, Fermentative hydrogen production using lignocellulose biomass: an overview of pre-treatment methods, inhibitor effects and

detoxification experiences, Renewable and Sustainable Energy Reviews. 77(2017) 28-42.

17. J.F. Soares, T.C. Confortin, I. Todero, F.D. Mayer, and M.A. Mazutti, Dark fermentative biohydrogen production from lignocellulosic biomass: technological challenges and future prospects, Renewable and Sustainable Energy Reviews. 117(2020) 109484.
18. S. Manish, K. Venkatesh, and R. Banerjee, Metabolic flux analysis of biological hydrogen production by Escherichia coli, International journal of hydrogen energy. 32(16) (2007) 3820-3830.
19. T.B. Madavi, V. Madathil, V.M. Aishwarya, K.-Y. Choi, S. Chauhan, and S.D.V.N. Pamidimarri, In- silico guided tailoring of whole cells exploring the Genome-scale metabolic model for production of bio-hydrogen using lignocellulose derived sugars: Prioritizing higher growth rate and productivity, International Journal of Hydrogen Energy. 177(2025) 151586, https://doi.org/10.1016/j.ijhydene.2025.151586.
20. P. Vilaça, P. Maia, H. Giesteira, I. Rocha, and M. Rocha, Analyzing and designing cell factories with OptFlux, in Metabolic Network Reconstruction and Modeling: Methods and Protocols. 2017, Springer. p. 37-76.
21. I. Rocha, P. Maia, P. Evangelista, P. Vilaça, S. Soares, J.P. Pinto, J. Nielsen, K.R. Patil, E.C. Ferreira, and M. Rocha, OptFlux: an open-source software platform for in silico metabolic engineering, BMC systems biology. 4(1) (2010) 45.
22. Y. Jiang, B. Chen, C. Duan, B. Sun, J. Yang, and S. Yang, Multigene editing in the Escherichia coli genome via the CRISPR-Cas9 system, Applied and environmental microbiology. 81(7) (2015) 2506-2514.
23. K.S. Kim SeongKeun, S.W. Seong WonJae, H.G. Han GuiHwan, L.D. Lee DaeHee, and L.S. Lee SeungGoo, CRISPR interference-guided multiplex repression of endogenous competing pathway genes for redirecting metabolic flux in Escherichia coli, (2017).
24. C.J. Hartline, A.C. Schmitz, Y. Han, and F. Zhang, Dynamic control in metabolic engineering: theories, tools, and applications, Metabolic engineering. 63(2021) 126-140.