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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

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ABSTRACT

In-silico guided biohydrogen production capability of mutants coupled with the effect on growth was studied under glucose and synthetically made lignocellulosic sugar mix, and growth temperatures. It was observed that simulations of gene disruption under two different sugar substrates (glucose and xylose) using *i*ML1515 Genome-scale metabolic model of *E. coli* successfully predicted formate and biomass production capabilities of the mutants. *In-silico* simulations showed succinate being dominantly co-produced with formate instead of lactate. Laboratory-developed mutants were scrutinized for their growth patterns and bH_2 production abilities under glucose or mixed sugars at 30 °C or 37 °C. Δ*adhE* was shown to produce the highest hydrogen (23.29 μmol H_2/mg protein H^{-1}) using mixed sugar as a carbon substrate at 30 °C. This is the first type of study, which explored the potential of *in-silico* guided metabolic engineering for the bH_2 production which supports the design-build-test strategy. This study establishes the direct relation between gene disruption and hampered growth rates; and temperature plays vital role, influencing hydrogen productivity.

1. Introduction

Soaring fuel demands coupled with environmental health deterioration are critical concern for the future global sustainability [1–3]. The counteractions involve relatively eco-friendly options of developing greener fuels, which will suffice future needs and will help to diminish the carbon emissions leading to improved environmental health [4]. Increasing demands for fuels have led to soaring levels of greenhouse gases affecting the climate [5,6]. In view of this, carbon-free fuel such as hydrogen produced by biological means has shown promising results, which can replace the traditional carbon-based fuels owing to its high energy density and no carbon emission qualities [7]. Hydrogen production is already commercialized using physicochemical processes, which are energy and cost-intensive, and the carbon neutrality is under scrutiny since it relies on fossil fuels [8]. To circumvent this problem, the cost efficiency of biological processes and exploring low-cost substrates as feedstock are now being preferred for hydrogen production [9,10]. Microbial cell systems are demonstrated to be the best catalytic base for the utilization of multiple waste biomass such as lignocellulose-derived sugars, crude glycerol, etc [11]. However, growth rates and cellular biomass are significant factors for the upscale and industrial-scale production. To get optimum productivity from the microbial systems, it is necessary to understand the cellular regulation of pathways and nodal metabolites with respect to bio-hydrogen (bH_2) production under dark fermentation.

Escherichia coli, a model facultative system, conducts hydrogen production using the Pyruvate Formate Lyase (PFL) pathway under anoxygenic conditions catalysed by the core enzymes, hydrogenases (Hyd) [12,13]. In *E. coli*, involvement of around 50 genes is said to define hydrogen metabolism [14]. Hydrogenase-1 and hydrogenase-2 show hydrogen uptake activity, and hydrogenase-3 and hydrogenase-4 are hydrogen-evolving enzymes [12,15,16]. Thus, studies focused on

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inactivating the former two hydrogenases report elevated hydrogen production. In *E. coli*, mixed acid fermentation results in the synthesis of acetate, ethanol, succinate and formate (Fig. 1) [17]. Formate is synthesised from pyruvate and the conversion is catalysed by the pyruvate formate lyase and further processed into hydrogen and carbon dioxide by formate-hydrogen lyase-3 (FHL-3) and formate-hydrogen lyase-4 (FHL-4). As the fermentation in *E. coli* leads to a mixture of products, which is a manifestation of carbon flux distribution among these pathways [18]. Thus, to increase the hydrogen production, carbon-flux is to be fed towards the pyruvate-formate lyase pathway by restricting the flux towards other by-products during fermentation [17]. The most sought strategy involves gene knockout of the enzyme that catalyses the by-product formation, inactivating the pathway and diverting the flux towards formate production [17]. In view of this, metabolic engineering was done to increase hydrogen productivity. Efforts were made towards eliminating the competing pathways of succinate and lactate, overexpression of the regulatory proteins and/or hydrogenases, eliminating the negative regulators, etc [19,20]. To increase the formate availability for FHL complex, Δ*focA* was studied and resulted in 14.85 μmol H_2/mg dry cell mass, yielding 0.63 mol H_2/mol glucose [14]. The approach has always been studying the single genes initially and analyzing their effect on biohydrogen production. Elimination of regulatory proteins, Δ*narL,* also resulted in 0.96 mol H_2/mol glucose but at the expense of diminished growth [14,17]. Diminished growth cannot support the optimum biomass production, and the decreased cell number also reduces the productivity [17]. The most critical mutations that are mandatory for increasing the hydrogen productivity in *E. coli* are Δ*hyaB* and Δ*hybC*, also known as uptake hydrogenases and their removal from the cell has been reported to have enhanced hydrogen production (Table 2). Lesser attention was paid to growth rates while creating mutants in the literature. Few studies emphasize the importance of the effect of growth on productivity. A study reports isolation of faster-growing mutant strain developed as a result of adaptive evolution grew 5-fold faster and produced 20-fold enhanced hydrogen [21]. In other aspects, for making the strains industrially feasible, they were engineered to utilize substrates

Table 1
List of genes and enzymes they code used for *in-silico* analysis.

Gene Name	Enzyme
frdC	Fumarate reductase subunit C
ldhA	D- lactate dehydrogenase A
pta	Phosphate acetyltransferase
ackA	Acetate kinase
adhE	Alcohol dehydrogenase (ethanol)
frdD	Fumarate reductase
poxB	Pyruvate oxidase
hyaB	Hydrogenase-1 large catalytic subunit
hybC	Hydrogenase-2 large catalytic subunit
focA	Probable formate transporter 1
focB	FocB protein
ppc	Phosphoenolpyruvate carboxylase
fdoG	Formate dehydrogenase-O major subunit
hyfG	Hydrogenase-4 component G
frdA	Fumarate reductase flavoprotein subunit
frdB	Fumarate reductase iron-sulfur subunit
adhP	Alcohol dehydrogenase
aceE	Pyruvate dehydrogenase E1 component

Table 2
Hydrogen yields for *E. coli* strains in sugar substrates depending on their genotypes.

E. coli strain	Genotype	Substrate	Hydrogen production	Reference
W3110	WT	Glucose	0.54 ± 0.04 mol/mol substrate	[14]
W3110	Δ*focA*	Glucose	0.63 ± 0.04 mol/mol substrate	[14]
W3110	Δ*hybC*	Glucose	0.7 ± 0.05 mol/mol substrate	[14]
W3110	Δ*ppc*	Glucose	0.73 ± 0.05 mol/mol substrate	[14]
BW25113	Δ*hybC*	Formate	7 ± 2 μmol mg-protein^{-1}	[74]
BW25114	Δ*hyaB*	Formate	1.6 ± 0.0 μmol mg-protein^{-1}	[74]

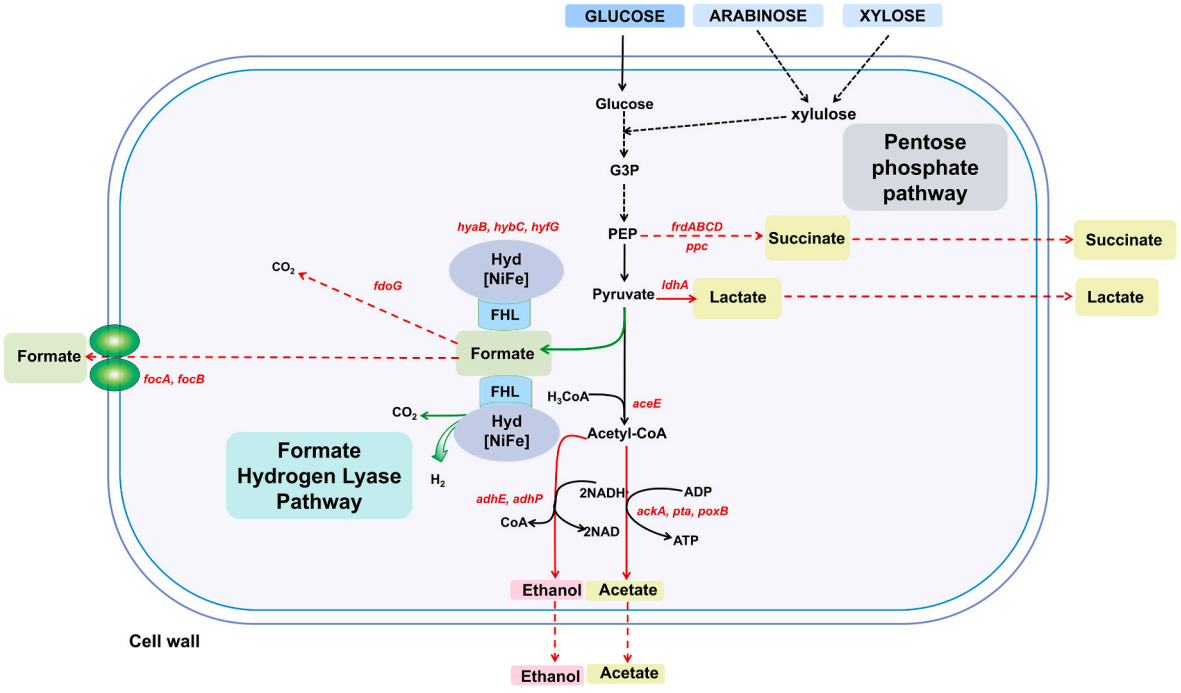

Fig. 1. Schematic diagram of pathways in *E. coli,* and genes involved highlighted in red. G3P, Glyceraldehyde-3-Phosphate; PEP, Phosphoenolpyruvate; Hyd, Hydrogenases; FHL, Formate hydrogen lyase; NADH, reduced Nicotinamide Adenine Dinucleotide; NAD, Nicotinamide Adenine Dinucleotide; ATP, Adenosine Triphosphate; ADP, Adenosine Diphosphate; CO_2, Carbon dioxide. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

that are low-cost and renewable, intending the technology to be sustainable for the future [22–24]. For any industrially feasible strain, there are two major concerns that should be addressed by the strain [25], it should have a good growth-coupled production capacity and [26], it should be able to grow using low-cost and sustainable raw materials with minimal ecological impact. *E. coli* has been extensively studied to grow on low-cost feedstocks like glycerol, industrial waste such as brewery spent grain, palm-oil mill effluent, paper industry waste and agricultural waste [27,28]. Where lignocellulose is being commonly pursued as it is a widely available renewable resource which can be used as a feedstock for *E. coli* fermentation for biohydrogen production [29]. In view of this, dark fermentation by *E. coli* for biohydrogen (bH_2) production has been proved as an efficient native producer [30].

Lignocellulose biomass is obtained as a result of indigenous forests and agricultural residues. Lignocellulosic biomass derived from agricultural wastes amounts to 140 billion metric tons per year [31]. As it is a waste resource and readily available in surplus over the globe, it poses as a good feedstock to be used for bioenergy production. This feedstock is recalcitrant due to its complex nature and thus needs to be converted into simplified components that can be reduced easily by microbes [32]. Lignocellulose-derived sugars, being abundant and renewable, can be used as a substrate for bH_2 production by *E. coli* [33]. However, bH_2 using lignocellulosic sugars faces significant challenges, such as low yield and a lack of efficient microbial strains. Whereas, developing a robust strain for high-yield is another challenge, stating its stability and scalability, keeping the growth rate intact [20,25]. The lignocellulose biomass composition varies depending on the source and pretreatment method used, which may influence the hydrogen productivity [34]. Reported studies have used synthetic compositions of lignocellulosic sugars such as glucose and xylose, with individual concentrations up to $10gL^{-1}$ for the fermentation and evaluated their effect on biohydrogen production [35]. A study also reports of yielding 0.9 mol H_2/mol sugars when synthetic lignocellulosic hydrolysates were used [36]. Growth rate equivalent to WT is a marker of successful process development of a metabolically engineered strain that reflects an optimum product assimilation. While engineering a strain for industrial applications, changes made at the subcellular level disturb the homeostasis, which takes time and resources to overcome these challenges. To develop such robust strain, a significant amount of resources and time is needed, which potentially increases the input cost of research and development. Addressing these potential challenges in the field of bH_2 production by *E. coli* is crucial for industrial applications and development.

Advancement of Genome-scale metabolic models (GEMs), curated based on the knowledge of omics, precisely replicate the gene-protein-reaction (GPR) associations in mathematical equations, and are now used to predict phenotypic states of an organism. Using these models and applying them to analyse or reconstruct metabolic pathways *insilico* has empowered the research by significantly reducing the operational time and resources [37,38]. In *E. coli*, a well-studied bacterial model organism, has already been used for production of various products, such as succinic acid, which was model-guided [39]. Earlier in the field of metabolic engineering, a handful of studies reported the use of methods developed for analysing metabolic fluxes in microbial cells. There are studies that involve hosts such as *Clostridium tyrobutyricum* and *Carboxydothermus hydrogenoformans* that demonstrate the application of such methods for hydrogen production [40,41]. And there are fewer studies that apply such methods in *E. coli* for analysing the hydrogen production [42], which encourages its further exploration. Our study provides a proof-of-concept of utilization of *E. coli* GEM, *i*ML1515, which is the most updated model which accounts for 1515 open reading frames and thus the name [43]. This model can successfully predict the physiology of an organism and data interpretation in a given condition. Using this powerful tool, we successfully demonstrate its application in identifying the genes that affect the hydrogen production and cellular growth. Here, hydrogen production was not directly assessed; instead, formate production was monitored, as model is constructed on the basis of C13 labelling and allows to track carbon containing moieties within the cell [44,45]. This model was operated with means of the OptFlux platform, which allows one to access the genes and reactions in the model to predict the effect of genetic perturbations on the model and co-relating it to the on-bench experiments saving the time and resources [46].

System and metabolic engineering of microbial cells require a robust and affordable method to modulate the metabolic pathway constitution to enhance the industrial level productivity. Discovery of CRISPR-Cas systems in prokaryotes and their applications have proved it to be a revolutionary method for genome editing [47]. CRISPR-Cas technology imparts advantages of using a single effector complex against various targets, as its specificity lies with the guide RNA (gRNA) [48]. These nucleases are programmed easily with a change in only gRNA and thus a dynamic method for modulating the metabolic phenotype [48]. CRISPR system overcomes the shortcomings of previously developed methods and tools, imparting affordability, higher efficiency, robustness, easy modulation and applications across various prokaryotic and eukaryotic systems [49]. Earlier methods developed, such as Cre-LoxP based methods, overexpression of heterologous gene/s, etc., and tools developed for genome editing, such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENS), etc. were tedious and time-consuming [50]. Therefore, a system was developed for dynamic application by repurposing the Type II CRISPR-Cas system from *Streptococcus pyogenes* as a recombineering method by complementing the λ-Red system with CRISPR for precise genome editing [51]. This allelic exchange system provides genome editing with precision, improved efficiency and easy to adapt techniques. Therefore, the CRISPR-Cas system with such advantages has proved to be a dynamic tool in the field of metabolic engineering and synthetic biology.

This study was done to explore the advantage of GEM models to assess the genotypic modulation of genes involved in the bH_2 production and its condition-dependent effect on growth rates upon removing the genes monitored for 18 genes using glucose or xylose as a substrate in anaerobic conditions (Fig. 1). And the promising genotypes that compete with the Pyruvate Formate Lyase (PFL) pathway for carbon flux, were demonstrated *in-vivo* to validate the proof-of-concept. This is the first report of using a GEM for assessing the growth-coupled hydrogen productivity, which was then verified by experimental genetic perturbations of 5 genes. Their growth pattern, substrate consumption and product portfolio were demonstrated to prove the alignment of *in-silico* and experimental setups.

2. Materials and methods

2.1. In-silico analysis metabolic models and simulating conditions

Genome scale model of *Escherichia coli, i*ML1515 was used for knockout and growth pattern studies in *in-silico*. JavaScript Object Notation (JSON) format of models was used for performing the simulations [43]. The models were used for simulating the single and multiple gene deletions by lowering the expression levels to zero for simulating knockout, and for repression, they were ranged between zero and one. Where one is the native expression of the respective gene/enzyme. All the simulations were performed while keeping the biomass reaction as an objective reaction. Parsimonious Flux Balance Analysis (pFBA) method was used for simulating wildtype and minimization of metabolic adjustment (MOMA) for mutants [46]. For anaerobic reactions, the uptake rate of oxygen (O_2 exchange) was kept zero. While simulating for xylose or arabinose as sole sugar substrates, the lower bound for glucose was kept zero, and for other sugars it was used as −10 mmol per gram cellular dry weight per hour (mmol/gDW/h) as reported for glucose uptake rate. No modifications were made in any of the models, and were used only for predictions. For predicting the growth while performing deletions and repression of genes in GEM, it was constrained to use respective sugar substrates by keeping the lower bound −10

mmol/gCDW/h. For simulating anaerobic conditions, oxygen uptake was constrained to zero by changing the lower bound value as suggested. For aerobic growth oxygen was unrestricted.

2.2. Bacterial strains, culture conditions and media compositions

The strains used in this study are DH5a, and Top 10 for cloning purpose. Wildtype MG1655 (K-12 F– λ– ilvG– rfb-50 rph-1) [from kind donation of Dr. Vijai, Indrasheel University] was used for all the studies for mutant generation and hydrogen production. Serum bottles were used for batch fermentations, capped with butyl rubber septa and sealed using aluminium caps/crimps. Sealed bottles were sparged with nitrogen for 15 min to maintain anaerobic conditions for fermentation. The bottles with a capacity of 125 mL and a working volume of 50 mL of media incubated at different temperatures (25–37 °C) at 180 rpm shaking conditions (LabWit Benchtop shaking incubator, ZWY-240, Shanghai, China), as required. Samples for growth, hydrogen and metabolite accumulation estimation were withdrawn at 24 Hr.

E. coli strains were streaked from glycerol stocks and grown on LB agar plate overnight at appropriate temperatures. For primary culture, strains were grown in Luria-Bertani broth (HiMedia Cat no.: M575-500G) overnight (OD 0.8). For secondary cultures, 2 % (from the primary culture) of volume for hydrogen production and growth curve experiments were inoculated. Experimental setups were all cultivated in the M9 (1X) media which was prepared using M9 (5X) salts from HiMedia (Cat. No: G013-500G) supplemented with yeast extract (HiMedia Cat no.: RM027-500G), $CaCl_2$ (Finar Cat no.: 10430SG500), $MgSO_4$ (HiMedia Cat no.: TC146-500G) and sugar substrates. Antibiotics used for mutant selection were kanamycin (50 mg/L), spectinomycin (50 mg/L), and ampicillin (100 mg/L) were added as required. Two different types of experimental media were used; (i) only glucose (8 g/L) was used as a sugar substrate as an experimental control and (ii) when mixed sugars comprising glucose (4.8 g/L), arabinose (0.4 g/L) and xylose (2.8 g/L) were used as a synthetic composition reflecting lignocellulose-derived sugar mixture.

2.3. Analytical techniques

Hydrogen gas was measured by gas chromatography, using Thermo Scientific TRACE 1610 gas chromatograph equipped with thermal conductivity detector (GC-TCD) (Trace 1600 series Italy). 500 μL of gas was injected using gas-tight syringe. Standard curve of the hydrogen gas for calibration was plotted with concentrations of 100, 5000, 50000, 100000, 250000 ppm. Argon was used as a carrier gas. Chromatograms were acquired and analysed using Chromeleon-7 software. The gas estimated was converted into μmol concentrations considering the headspace of the culture serum bottles in volume (mL), and molar concentrations were calculated according to the Avogadro's Law for gases occupying the same volume at standard temperature and pressure using following formula;

$$\boldsymbol{\mu mol\ H_2\ in\ headspace = mL\ hydrogen\ in\ headspace * 446.428}$$

Sugars (glucose, arabinose and xylose), organic acids (acetic acid, lactic acid, formic acid and succinic acid) and ethanol was determined using High Performance Liquid Chromatography (HPLC). Supernatant was filtered through a 0.22 μm PVDF syringe filter and was injected into the column. Shimadzu HPLC system (N series) equipped with a refractive index detector RID-20A (Japan) used with an Aminex column (HPX-87H, Bio Rad, USA) with dimensions of 300 × 7.8 mm, incubated at 65 °C. Isocratic solvent of 5 mM sulfuric acid (HPLC grade Sigma cat. no. 7664-93-9) was used as an eluent at a flow rate of 0.6 mL/min with total run time of 30 min. LabSolutions software was used for data processing. Biorad organic acid standards (Catalogue# 125-0586) were resuspended in 1 mL autoclaved milliQ, having concentrations of 40 μmol/mL of sodium acetate, and 20 μmol/mL of sodium succinate and sodium formate each. Standards were plotted using concentrations 0.2, 0.5, 1, 2, 4 and 8 % for organic acids and ethanol; whereas, standards were plotted against the concentrations of 0.1,0.5, 1, 2, 4 and 8 g/L for sugars.

Total protein of cell biomass after 24 h was estimated using the Bradford method. The sample was centrifuged at 5000 rpm for 10 min at 4 °C and, the supernatant was discarded. The pellet was washed twice with chilled 1X phosphate buffer solution (PBS), and the pellet was processed further for sonication. Washed pellet was resuspended in 2 mL PBS and sonicated with 15sec pulse on and 5 sec pulse off until a clear solution is observed. 10 μL of sonicated sample or standard was added to Bradford solution and was incubated in the dark for 15 min, and was then read at 595 nm. Standards were prepared with bovine serum albumin (BSA) at concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1 mg/mL and were plotted to derive the line equation which was then solved to calculate the concentration of protein in a concentrated 2 mL pellet. Protein concentration observed in 2 mL concentrate was extrapolated to the original 50 mL volume in mg.

Hydrogen productivity per mg protein per hour was then calculated according to the following equation for comparison with literature using following formula;

$$\boldsymbol{H_2\ productivity = (\mu mol\ H_2\ in\ headspace)/(total\ protein\ in\ mg * 24)}$$

Biotek Cytatio-5 multimode reader (USA) equipped with Gen-5 software was used for data capture and analysis of cell growth and protein estimations.

2.4. Genetic modifications and cloning strategy

For genetic modification, a two-plasmid system was used with plasmids p-TargetF (consisting of N_{20} sequence, sgRNA sequence) and with co-expression of plasmid containing Cas9, pCas. The sgRNAs were selected with the help of an online resource named as CRISPy-web (CRISPy-web) [52] and were cloned using inverse PCR with overlapping regions of 18-20bp in pTargetF. Supplementary Table S1 depicts the N_{20} with respective gene sequences selected for study. Donor DNA was designed to have flanking homologous regions to target site for occlusion of the N_{20}. Homologous regions flanking the target site were situated 60bp away at each end so that the mutant will exhibit an occlusion of 140 bp, which could be differentiated from the WT, giving ~540bp (Supplementary Fig. S1). Donor bricks consisting of flanking homologous regions for the target genes were cloned into pUC18 for ease of repeated amplification. All sgRNAs were under the regulation of their individual promoters. Primers used for amplifying donor DNA are listed in Supplementary Table S5, and designed sgRNA are listed in Supplementary Table S2. All the plasmids were maintained in Top10 *E. coli* strain grown on LB plate with the respective antibiotic for selection. MG1655 competent cells were primarily transformed with pCas to express λ-Red recombinases by inducing it with 10 mM arabinose. Secondary transformation of λ-Red induced competent cells was co-transformed with the pTarget-F containing sgRNA and purified linear donor brick in the ratio of 1:4 of respective target gene to cure the double strand break by recombining it with homologous strands. These transformations were performed at 30 °C, as pCas is temperature sensitive, and thus mutants were cured of both plasmids after successful perturbation by incubating them at 42 °C overnight, devoid of any antibiotic selection. Cured mutants were then carefully isolated and preserved as a glycerol stock in −80 °C. Cured mutants were then used for experimental analysis by streaking them freshly on LB agar plate as needed. Knockout strategy used in this study is briefed in the Supplementary Fig. S3.

Primers were procured from Barcode Biosciences, Bangalore. Ultra One Step Cloning kit was procured from GENES2ME (catalogue# UOSC-50rxn) for PCR amplified product cloning. Plasmids were isolated using Qiaprep spin miniprep kit from QiaGen (catalogue# 27104). Mutants were verified on 1 % agarose gel by electrophoresis. List of sgRNA embedded primers is available in Supplementary Table S2 pTargetF (62226) and pCas (62225) were procured from Addgene.

2.5. Statistical analyses

All the experiments were performed in triplicates. Variations between the experimental data points and statistical analyses were done using Microsoft Excel.

3. Results and discussion

E. coli is an efficient system in utilizing various sugar substrates and flourishes in dynamic environmental conditions. Many studies were made to inactivate the uptake hydrogenases, FhlA repressor, and formate dehydrogenase-O, restricting the consumption of produced hydrogen majorly and certainly increased hydrogen production when glucose was fermented [53]. Formate is another, but rather expensive, substrate which was used for the hydrogen production and gives better results over glucose as it is already a reduced product of the glycolytic pathway and is directly catalysed by the Formate hydrogen lyase (FHL) complex comprising formate dehydrogenase, hydrogenase, electron-transfer proteins and anchor proteins [53]. Thus, lignocellulosic biomass and its hydrolysates were sought as an inexpensive sugar substrate for hydrogen production, which satisfies the criteria of being a low-cost substrate. Lignocellulosic waste such as wheat-straw hydrolysate has been reported to yield bH_2 which could be scalable in future [23]. Such examples encourage to pursue sustainable waste biomass as a substrate for hydrogen production. Designing cell factories for production at industrial scale depends on a great amount of 'design-build-test" cycle which increases the time and cost of resources invested. To bypass this cycle, systems and synthetic biology played a vital role in developing mathematical models that depict the complex cellular pathways and their interactions resulting in a rational phenotypic trait.

These GEMs are powerful tools and are available for all model organisms on databases such as the Biochemical Genetic and Genomic (BiGG) knowledge-base [54]. *i*ML1515 is the most complete GEM of *E. coli* MG1655 K-12 strain, which can be worked on with constraint-based algorithms that enables engineering of microbial strains. This is a feasible method for identifying and strategizing an effective combination of genes and their modulation for growth-coupled product formation. As the metabolic engineering involves intricate and balanced modulation of native genes by regulating on/off their activity to increase the production while maintaining the cellular health [38].

This work demonstrates the capability of GEM predictions for a biomolecule production/accumulation, and it can simulate the cellular reactions to give the optimum results. For bH_2 production, efforts were made to make its process industrially sustainable in view of low-cost input coupled with maximum output as a non-compromised growth rate of the cell. GEM used for this study is *i*ML1515, as it was reconstructed utilizing various sugar substrates such as glucose, fructose, ribose, xylose, etc. Considering lignocellulosic sugars as a substrate for the experimental setup, utilizing the prediction capability of GEMs, we screened for the genes which influence the formate production in *E. coli* (Table 1). As formate is a terminal carbon-containing molecule in the Pyruvate-formate lyase pathway and is directly proportional to the hydrogen produced. Hence, to save the time and resources, we performed simulations using *i*ML1515, carefully curated by assessing the multi-omics approach and GPR connections, proving it to be a powerful tool for mathematical predictions. We used glucose and xylose as sugar substrates to simulate the hydrogen production to reflect lignocellulose-derived sugars, which mainly comprises of glucose, xylose and arabinose. As arabinose and xylose are pentoses and undergo the same metabolic pathway, here only xylose was used, and moreover, *i*ML1515 is optimized using xylose but not arabinose. We also monitored the growth and product formation on-bench to verify the predictions and as a proof-of-concept for that the growth-based strain selection is a better alternative to that of product-based, here bH_2. This study shows the utility of GEMs developed for predicting the production of hydrogen and also adds to the existing knowledge of GEM, which will help it to be developed for the hydrogen production.

3.1. Predicting the formate productivity and biomass

The *i*ML1515 model developed using 'omics' data does not include the H_2 molecule; hence, in the analysis the penultimate substrate of the FHL complex is taken as a reference and assumed to be proportional to the H_2 evolution from the cell. Hence, we used glucose or xylose as a sugar substrate for assessing the formate production capability of *E. coli*. Genes that are related to pathways that compete with the FHL pathway or influence the formate accumulation in the cell were targeted. Gene essentiality of 18 genes (Table 1) was assessed while monitoring their effect on the byproduct accumulation in anaerobic conditions, with special reference to formate accumulation. While the genes were targeted, conserving the cellular growth and cell biomass generation is crucial for future upscaling studies. Hence, another factor monitored was the effect on biomass production during the assessment of gene deletion. Single gene inactivation studies using glucose as a substrate in anaerobic conditions revealed that inactivation of *ackA* (acetate kinase catalyses acetyl-phosphate to acetate) produced 100.0201 % formate, which was equivalent to the WT. Although the formate production was not hampered significantly but biomass was reduced to 54.41806 % of WT (Fig. 2a). Inactivation of formate transporters *focA* and *focB* resulted in the least formate production of 50.05001 % each, rendering them as relatively essential for cell survival, as their inactivation resulted in zero biomass production. As there are studies that report the inactivation of *focA* and *focB* transporters allegedly increases hydrogen production by restricting its excretion from the cell, making all the formate available for formate hydrogen lyases [55]. Intracellular accumulation of formate and being an electron donor, its presence in appropriate amounts is balanced by the transporters, and thus, inactivating them can uncouple the membrane potential, affecting the cellular growth [56]. Along with *focA* and *focB,* other genes namely *pta*, *adhE* and *adhP,* also showed significant decline in the growth of cell as biomass of these inactivation resulted into 9.972318 %, $6.2*10^{-10}$ % (negligible) and $-4.7*10^{-08}$ % (negligible) of WT, respectively, which is a very undesirable for the bH_2 production (Fig. 2a) [57]. This result is justifiable as ethanol is one of the major byproducts of the fermentation in *E. coli* where it maintains the redox balance in cell and *adhE* mutants are reported to not grow in sugar such as glucose [30,58]. As *E. coli* produces formate and acetyl CoA when pyruvate is split, the acetyl CoA is then converted into either acetate or ethanol based on the redox status of the cell [59]. Thus, removal of genes involved in the acetate or ethanol production might affect the cellular homeostasis. Moreover, these genes affected the biomass production, inactivation of all other genes produced formate fairly enough compared to the WT apart from *focA* and *focB*. As formate transporters play a vital role in maintaining membrane potential through formate concentrations across the cell membrane, and their removal or inactivation resulted in the affected biomass as cellular death is anticipated due to increased intracellular formate concentrations causing toxicity [14,56].

We also observed an increase in succinate production upon inactivation of genes *focA*, *focB*, *pta*, *adhE* and *adhP* as a proof of the cellular flux diversion towards reducing succinate at higher concentrations (Fig. 2g). Lactate production was observed in case of deletions of genes such as *focA*, *focB*, *ackA*, *adhE* and *adhP* only, which states that lactate is not primarily produced during anaerobic respiration and is rather a product of conditional gene disruption. This was necessary to note that there are studies which have used gene combinations that involve the *ldhA* gene deletion for metabolic engineering studies to increase hydrogen production in *E. coli* [19,60]. This can be corroborated by other studies as acetate and ethanol are the primary products of dark fermentation in *E. coli* and hence eliminating genes catalysing the formation of these products will divert the flux towards lactate and/or succinate production to maintain the cellular homeostasis [61] (Fig. 2c and e). As these genes were scrutinized for their essentiality for cell

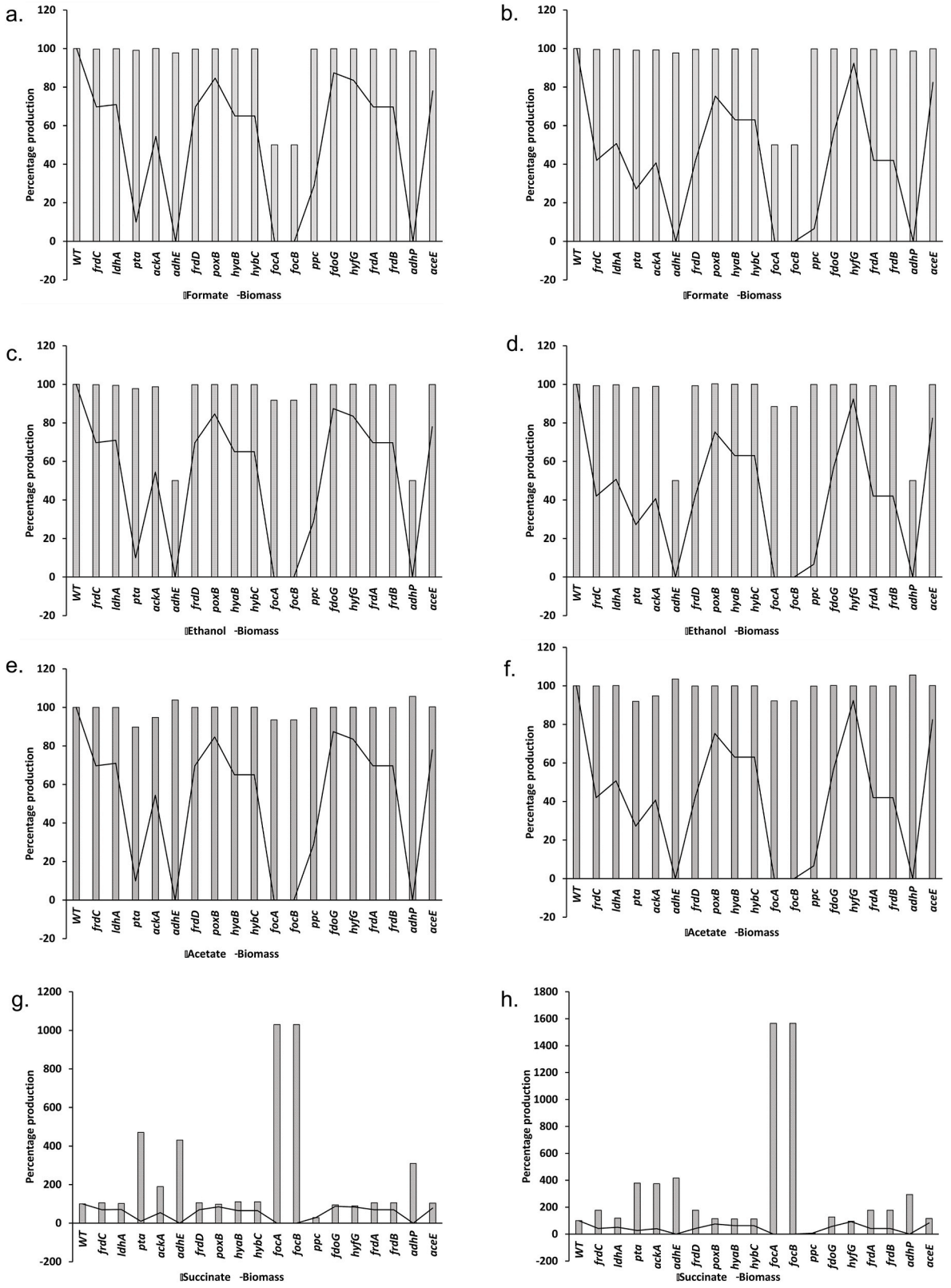

Fig. 2. Product (bars) portfolio of *in-silico* simulated mutants with biomass (line graph). Results are depicted in terms of percentage production with respect to WT being a 100 %; (a) formate and biomass production in glucose as a substrate, (b) formate and biomass production in xylose as a substrate, (c) ethanol and biomass production in glucose as a substrate, (d) ethanol and biomass production in xylose as a substrate, (e) acetate and biomass production in glucose as a substrate, (f) acetate and biomass production in xylose as a substrate, (g) succinate and biomass production in glucose as a substrate, (h) succinate and biomass production in xylose as a substrate.

survival to meet the optimum biomass and formate production, they were also studied for the product portfolio to analyse the effect of deletion on the cellular homeostasis. Glucose, being the primary sugar metabolized by *E. coli*, is involved in the logarithmic phase of the cellular growth, and thus much of the cellular biomass is dependent on the glucose concentrations available in the media.

We also performed simulations using xylose as a sugar substrate in anaerobic conditions. Xylose, being a pentose sugar does not follow glycolytic pathway but it is metabolized through the pentose phosphate pathway, which results in fructose-6-phosphate and is fed in the glycolytic pathway for pyruvate production [61]. Simulation study using xylose as a substrate resulted in similar observations as discussed above for glucose. Where, *focA* and *focB* deletion reduced formate production to 50.05 % each, and resulting in no biomass stating the unsuitability for deletion studies. Apart from formate transporters, other genes under scrutiny performed similarly with formate production up to 99.89 % (*aceE*) of WT (Fig. 2b). But biomass is affected if *adhE*, *ppc* and *aceE* are eliminated, which resulted in the biomass production of $-4.7*10^{-26}$ % (negligible), 6.598026 % and $2.79*10^{-09}$ % (negligible) of WT, respectively. Following to this, ethanol production is reduced when *adhE* and *adhP* are eliminated producing 50.04 % ethanol of WT, as these genes correspond to alcohol dehydrogenases which catalyses the ethanol production (Fig. 2d). Additionally, these deletions increased the acetate production (103.6078 and 105.6146 % of WT, respectively) as a compensation of reduced ethanol production (Fig. 2f). It was also observed that the ethanol production was increased in the case of *poxB* and hydrogenases (*hyaB* and *hybC*) deletion. As carbon flux was diverted to ethanol production due to the elimination of hydrogenases, the cellular homeostasis supports redox balance and energy generation by reducing acetaldehyde to ethanol. Whereas, succinate production had a similar pattern when glucose was taken as a substrate.

As glucose is the preferred sugar for metabolism in *E. coli* and biomass production, is a primary driver of the gene selection for forging further strategies of improving hydrogen production. This study helped in understanding the prediction capability of GEM *i*ML1515 for assessing the growth-coupled biomass production. Gene deletion and their respective byproduct portfolio suggest a well-balanced cellular circuit that maintains the redox balance and biomass. These GEM based simulations predict the cellular behaviour after a perturbation made in the genome, which is helpful in strategizing a framework for metabolic engineering strategies. Although, the GEM could simulate using a single sugar at a time, on-bench results may vary as lignocellulose consists of different hexose and pentoses and the cell follows the carbon-catabolite repression while utilizing those sugars. Also, the growth rate is influenced by the CCR and therefore, GEMs are restricted to simulate the biomass growth at a single sugar, which differs from real metabolism, especially when lignocellulose derived sugars are studied. Due to limitations of GEM predicting capability under mixture of sugar substrates, we considered to perform *in-vivo* gene deletion studies to verify the effect of secondary sugar on cell phenotype. Considering growth conditions and sugar concentrations present in the lignocellulose, glucose being the primary and most abundant sugar present, genes *hyaB*, *hybC*, *adhE*, *ackA* and *frdC* were further selected for *in-vivo* gene deletion strategy to monitor their individual effect on hydrogen production. Among genes listed to be studied *in-vivo*, *hyaB* and *hybC* code for the large subunit of hydrogenase 1 (Hyd1) and hydrogenase 2 (Hyd2), respectively. These hydrogenases reversibly consume the hydrogen produced by the cell and thus need to be deactivated [62]. There are studies reporting the elevated hydrogen concentrations when Hyd1 and Hyd2 were deactivated (Table 2) [14,63–65]. Whereas, *frdC*, *ackA* and *adhE* are involved in the production pathways of succinate, acetate and ethanol, respectively. These genes were studied based on their simulation studies as discussed above and they are involved in the key fermentation product formation that competes with the FHL pathway for carbon flux. We chose these genes as they were the primary and crucial targets to avoid the competition for carbon flux. And this study is a demonstration of GEM application rendering its ability of predicting metabolic phenotype as a result of perturbation.

3.2. Growth pattern across sugar substrates and temperatures

For validating the simulations, we performed the gene disruptions in wildtype *E. coli* MG1655-K12. Instead of knocking out the complete gene, we disrupted gene expression of structural protein using CRISPR-Cas9 technology by occluding 140bp and introducing stop codons instead (Supplementary Fig. S3). As reported by Kurokawa et al., that reduction in size of the genome affects the growth of the cell, we suspected the same phenomenon to occur when genes are totally removed or knocked out from the genome, affecting the growth of the cell [25]. As growth is an important factor for any engineered strain to be used for industrial applications, incubation temperature also influences the growth and also might have effects on hydrogen production [66]. Thus, we harnessed the CRISPR-Cas9 technology complementing to the λ-red recombineering system and disrupted the target gene to make it non-functional, posing as a knockout [67]. This gives an advantage over the problem of decreased growth rate of the cell after perturbation in the genome. Effect of temperature and sugar substrate concentrations on bH_2 production was widely studied in the past and are vital factors to be scrutinized in this study [68]. Glucose is a primary sugar substrate used by *E. coli,* hence it was used as a control since many studies were reported using this substrate as a default sugar [69–71]. Disruption of genes resulted in similar growth patterns as that of the WT strain (Supplementary Fig. S2). Single gene disruption, when studied anaerobically using glucose as a substrate, the WT showed specific growth rate of 0.105 H^{-1} at 30 °C, which was the highest as cell was at its native optimal (Fig. 3a). As we were aiming to track the growth profiles of mutants to align with the WT, expecting least perturbed growth rate aimed to have optimum biomass and bH_2 production. The growth rate was least in the case of Δ*frdC*, 0.077 H^{-1} at 30 °C, which was in correspondence to *in-silico* results which showed succinate to be one of the primary products of fermentation and thus its removal will affect the growth of the cell (Fig. 3a; Table 4) [18]. Whereas, temperature may affect the growth rate of the cell, as it was observed that Δ*frdC* showed the least among all the mutants as compared to the WT when grown at 30 °C and 37 °C with growth of 0.077 H^{-1} and 0.101 H^{-1}, respectively (Table 4). This effect of temperature on growth rate fuelled the curiosity to study its effect on bH_2 as well, which is discussed later in this paper. Δ*hyaB* grew very slowly with a very long lag phase across both at 30 °C and 37 °C, this could be due to the disruption in cellular homeostasis anaerobically, as *hyaB* codes for the large subunit of hydrogenases−1 [72] (Supplementary Fig. S2). Though a longer lag phase was observed in the case of Δ*hyaB,* a growth rate of 0.29H^{-1} was observed when grown at 37 °C in the presence of glucose, and a log phase was observed after 20 h of inoculation (Supplementary Fig. S2a). Similar observations were documented by Poladyan et al., stating slow growth with longer lag phases on minimal media (minimal salt medium) compared to peptone media, with 3-fold decrease in growth [68]. A longer lag phase will demand for elevated cost input at an industrial scale and is undesirable for bH_2 production if the problem persists. This hydrogenase-1 is reversible in nature and disruption of its function may lead to suppressed cellular viability. This may be due to the uncoupling of hydrogen oxidation, as Hyd1 is involved in doing so for energy conservation in anaerobic conditions since hydrogen also acts as an electron donor. As glucose being a costly option and effect of the temperature on the growth of mutants laid a basic foundation of understanding; and thus, we also studied growth patterns of these mutants in a synthetic composition of lignocellulosic sugars (now on will be termed as mixed sugars), such as glucose, xylose and arabinose, which are the three major sugars can be obtained upon hydrolysis of lignocellulose.

Similar growth studies were also conducted with the single gene disruption mutants in mixed sugars in anaerobic conditions at 30 °C and 37 °C. Where the Δ*hyaB* grew at the slowest rate with a specific growth

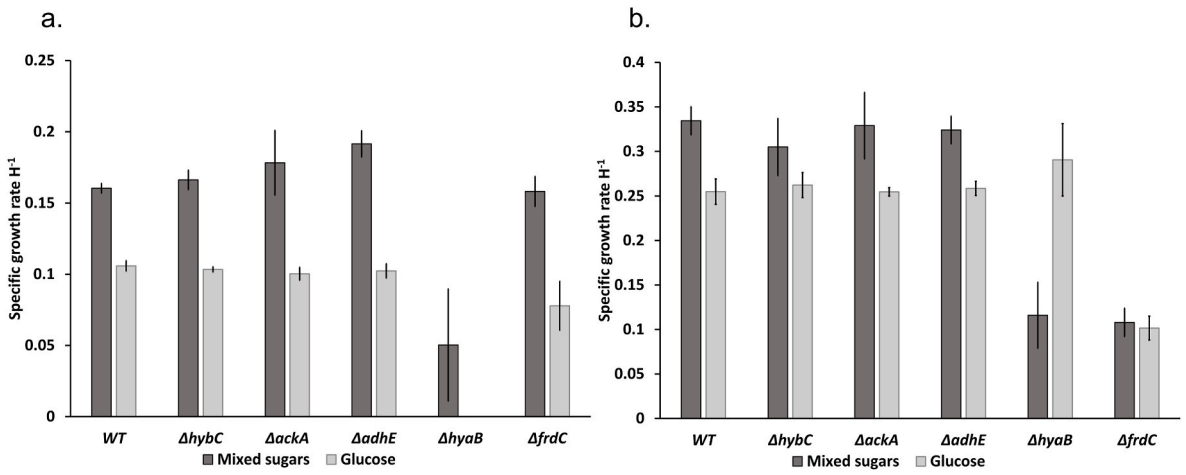

Fig. 3. Specific growth rates of mutants and WT (control) in anaerobic conditions either in glucose or mixed sugars. (a) specific growth rates of mutants when grown at 30 °C and, (b) specific growth rates of mutants when grown at 37 °C.

Table 3
Lignocellulose biomass hydrolysates, their components and type of pretreatment employed.

Biomass	Type of pretreatment	Concentrations in gL^{-1}			Reference
		Glucose	Arabinose	Xylose	
Wheat straw	Dilute acid	1.54	1.93	13.96	[23]
Rice straw	Dilute acid	16.22	–	32.46	[86]
Rice straw	Enzymatic	66.17	–	10.25	[86]
Sugarcane bagasse	Enzymatic	51.3	–	17.8	[87]
Wheat straw	Enzymatic	75.3	6.4	38.2	[88]
Corn stover	Enzymatic	68	5	24.2	[88]

Table 4
Growth rates of *E. coli* mutants grown anaerobically from this study depending on their culture conditions.

Genotype	Growth rates (30 °C)		Growth rates (37 °C)	
	Mixed sugars	Glucose	Mixed sugars	Glucose
WT	0.160 ± 0.003	0.105 ± 0.003	0.334 ± 0.016	0.254 ± 0.014
Δ*hybC*	0.166 ± 0.006	0.103 ± 0.00	0.304 ± 0.032	0.262 ± 0.014
Δ*ackA*	0.178 ± 0.02	0.100 ± 0.004	0.328 ± 0.037	0.254 ± 0.004
Δ*adhE*	0.191 ± 0.009	0.102 ± 0.005	0.323 ± 0.015	0.258 ± 0.007
Δ*hyaB*	0.050 ± 0.03	0.000	0.115 ± 0.037	0.290 ± 0.04
Δ*frdC*	0.158 ± 0.01	0.077 ± 0.017	0.107 ± 0.016	0.101 ± 0.01

rate of 0.05 H^{-1} and 0.115 H^{-1} in anaerobic conditions at 30 °C and 37 °C, respectively. Alike previously discussed growth of Δ*hyaB* in glucose, slower growth was observed in mixed sugars as well, at both 30 °C and 37 °C as compared to the WT (Supplementary Fig. S2). Moreover, as discussed earlier that disruption of genes could lead to the slower growth of the cell, contradictory to which we observed an increased rate in Δ*adhE* (0.191H^{-1}) followed by Δ*ackA* (0.178 H^{-1}) and Δ*hybC* (0.166 H^{-1}) when grown in mixed sugars at 30 °C (Fig. 3; Table 4). There are studies done using lignocellulosic hydrolysates, synthetic mixtures representing the lignocellulosic sugar composition or individual pentoses and hexoses as a carbon substrate to understand the cellular behaviour under the regime of complex media, so the optimization studies could be framed [22,65,68,73]. As our overall study focuses in adopting lignocellulose derived sugars for the hydrogen production, we emphasised studying the mutants in mixed sugar substrates at both 30 °C and 37 °C. Where WT grown in mixed sugars resulted in the 0.334 H^{-1} and was highest compared to all the mutants, although mutants showed relatively equivalent growth rates as that of WT except Δ*hyaB* at 30 °C and Δ*frdC* at 37 °C [74]. Thus, comparing the results in both types of sugar substrates at 30 °C and 37 °C, mutants grown in mixed sugars and at 30 °C align with the GEM predictions while stating the importance of temperature on the cellular growth, which is an important factor in hydrogen production and influences it significantly. GEMs are designed to operate at a single native optimal temperature of the organism and might be unable to replicate the dynamic cellular behaviour at variable temperatures.

Although the studies reported glucose as the preferable sugar, however consumption of low-cost substrates such as lignocellulose-derived sugars in the case of upscale production context could be a futuristic option [22]. Hence, this study concludes that using the mixed sugars as a more preferred option as per the cellular homeostasis and evident from the growth observed in mutants across both types of substrates and temperatures. Growth studies elaborate the suitability of mutants for further consideration in production as it emphasizes on the cellular behaviour over time and feasibility for further studies. Considering the overall growth pattern of all disruptions studied under different cultivating conditions, it was concluded that though glucose is the most preferable sugar for metabolism, secondary sugars might help for the cellular growth, maintaining an optimum biomass for the late log-phase. The synergistic effect was observed when mixed sugars were used as a substrate for growth, which was then used primarily for further hydrogen production studies.

3.3. Hydrogen production in mutants

Hydrogen production was monitored for all the mutants at 30 °C and 37 °C when grown in glucose or mixed sugars as a carbon feedstock for 24 h. As hypothesised, the relation between biomass generation and hydrogen productivity, it was important to understand the cellular behaviour in terms of hydrogen production with respect to different temperatures and sugar substrates. We expected elevated hydrogen concentrations in mutants with higher growth rate and shorter lag phase (Supplementary Fig. S2). Earlier studies for hydrogen productivity of single gene knockouts were assessed in *E. coli* for genes such as *hyaB*, *hybC*, *hycA*, *focA*, *narL*, etc. (Table 2), using formate or simple sugars as a substrate, which certainly helped in understanding their roles in bH_2 production [14,74]. This encouraged us to experimentally assess the hydrogen productivity of our mutants, especially when grown on mixed sugars. Our study shows mutant Δ*adhE* exhibits the highest hydrogen productivity in mixed sugar when grown at 30 °C, which was 23.291 μmol H_2/mg protein H^{-1} (Fig. 4a). This corresponds to the highest growth rate as discussed earlier corroborating the relationship between optimum biomass and increased hydrogen production at 30 °C (section

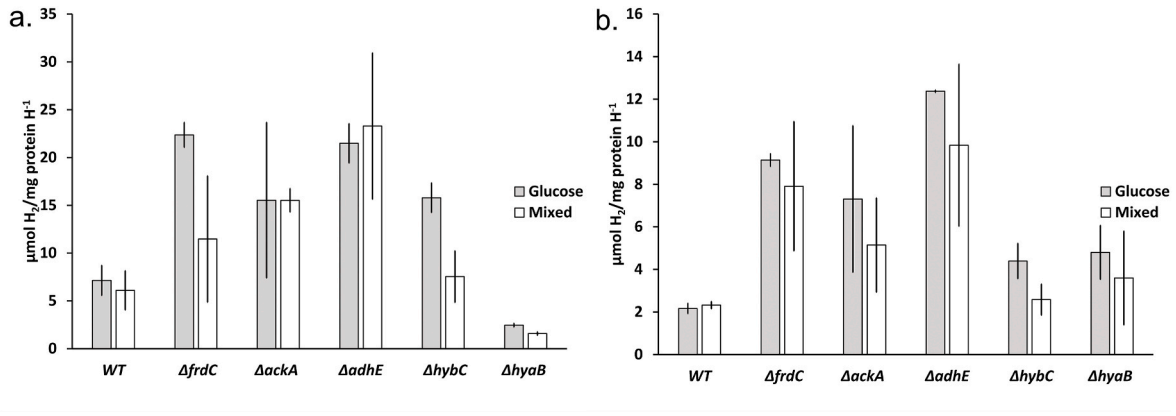

Fig. 4. Hydrogen productivity of mutants and WT (control) in anaerobic conditions either in glucose or mixed sugars, (a) when grown at 30 °C and, (b) when grown at 37 °C.

3.2). This was important to note, as lignocellulose-derived sugars are primarily sought for biohydrogen production owing to its low-cost and sustainability, and also this study encourages the same [22,75]. The growth studies also reveal that lowering temperature might help with slower cellular functions, helping with time to attain homeostasis after perturbed genetics and its metabolic effect (Supplementary Fig. S2c and S2d). Alike Δ*adhE,* Δ*frdC* produced 22.366 µmol H_2/mg protein H^{-1} when grown at 30 °C using glucose as a substrate (Fig. 4a). This shows that sugar substrates may influence the productivity of *E. coli*, as mixed sugars encourage the diauxic growth coupled with production stage onsets later in the log phase, transitioning to the stationary phase as a result of pH drop in anaerobic conditions (Supplementary Fig. S2d).

There are various studies done for enhancing the hydrogen productivity in *E. coli* as it is a model organism using lignocellulose derived sugars [22]. Effect of individual gene disruptions are also reported to have an effect on cell biomass and hydrogen production, and in our initial studies, it is evident (Fig. 3a and b). The hydrogen yield from literature for Δ*hybC* was found to be 1.4 µmol H_2/mg protein H^{-1} and 7 µmol H_2/mg protein H^{-1} when grown on glucose and formate, respectively [14,65]. Our study reports overall relatively higher yield as compared to previous documentations. There are studies which report inactivation of individual genes and their effects on the hydrogen productivity when grown in glucose but lesser attention was paid on the biomass [74]. Optimum biomass with growth rate closer or equivalent to WT produced by an individually inactivated gene is directly proportional to the increased hydrogen production rates which is evident from the Δ*adhE*. But, in this study we also observed that temperature also plays an important role, similar effect was seen in growth studies (Supplementary Fig. S2). As observed growth and the respective hydrogen producing capacities of mutants at 30 °C which was better than that at 37 °C (Fig. 4a and Supplementary Fig. S2d). As seen in case of 37 °C when compared among all the mutants grown in glucose or mixed sugars, the highest hydrogen produced was reported to be the 12.37 µmol H_2/mg protein H^{-1} when grown in glucose (Fig. 4b). Whereas, mutants grown at 30 °C in glucose or mixed sugars, Δ*adhE* mutant resulted in highest productivity with 23.29 µmol H_2/mg protein H^{-1} when grown in mixed sugars. The least production of hydrogen was observed in case of Δ*hyaB* at 30 °C (1.583 µmol H_2/mg protein H^{-1}) when grown in mixed sugars and similar trend was observed in case of glucose as well. Least hydrogen production was observed in Δ*hyaB* with production rates of 2.455 µmol H_2/mg protein H^{-1}. which was even lower than WT. This might be the manifestation of longer lag phase as a result of the disturbed cellular homeostasis and time required to acclimatize to the niche which is evident from its growth pattern (Supplementary Fig. S2).

When mutants were grown at 37 °C in both glucose and mixed sugar substrates, overall productivity was less as compared to the 30 °C. Similar to 30 °C, Δ*adhE* produced maximum hydrogen relative to the WT when grown both at glucose or mixed sugar substrate. Among mutants, Δ*hyaB and* Δ*hybC,* lacking hydrogenases performed very poorly with hydrogen production as depicted in Fig. 4b. Effect of type of sugar, its concentration and temperature at which the cells are grown influence the biomass and hydrogen productivity. Realizing which, here we establish the direct relation between a better cellular health led to optimal product formation. This also infers that genomic perturbation affecting the cellular homeostasis slows down the metabolism. And cell needs time to adjust to the altered metabolic state and lower temperatures are said to have effect on expression of genes involved in energy metabolism [62,76] which might influence the growth. The cumulative effect of incubation temperature, perturbed pathway and cells adaptation to its intra- and extracellular changes might contribute to its slow growth with effect on product-oriented metabolism. This can be seen from overall better bH_2 productivity of mutants at 30 °C than at 37 °C, and also from Supplementary Fig. S2c and d, depicts the at par growth of mutants with WT. Studies report the change in protein constitution as a result of change in incubation temperatures which also influences the cell growth rate [77–79].

The yield of hydrogen was also estimated for all the mutants across all the culturing conditions studied. The mutants studied here produced hydrogen comparable to previous studies reported (Table 2). Studies reported in *E. coli* W3110 with Δ*hybC* genotype produced ~0.7 mol H_2/mol glucose which was ~1.3fold increase as compared to its wildtype (Table 2). Whereas, our study reports the ~1.5-fold increase in the Δ*hybC* mutant with 0.307 mol H_2/mol glucose and 0.246 mol H_2/mol glucose when grown at 30 °C in glucose and mixed sugar, respectively. Across all conditions studied, Δ*adhE* showed maximum yield from both types of sugar substrates used with 1.8-fold increase in hydrogen yield in mixed sugar at 30 °C (Fig. 6). As observed before in growth studies and hydrogen production, the yield in mixed sugars was relatively better than in glucose establishing the mixed sugar as a better substrate for biohydrogen production (Fig. 6). Δ*hyaB* resulted in the least yield (0.026 mol H_2/mol mixed sugar at 30 °C) across all conditions studied which also corroborates with previous observations in growth and productivity aspects. It codes for the large subunit of hydrogenase 1, an uptake hydrogenase which utilizes hydrogen produced in the cell in order to maintain the pH and availability of hydrogen [68]. As reported earlier by Maeda et al., the increase in hydrogen production in Δ*hybC* and production was decreased as a result of Δ*hyaB,* but their cumulative effect resulted in the 3.2-fold higher hydrogen production (Table 2) [65]. Similarly, our results testify the same results for single gene mutations (Fig. 6) and their combined effect increases the productivity of hydrogen (results not shown). This study infers the importance of studying the individual gene effects and their implications to frame strategy for the multigene interferences to increase the hydrogen yield.

Apart from Δ*adhE* mutant, Δ*ackA* and Δ*frdC* performed well in this study. Based on our *in-silico* analysis, mixed acid fermentation resulted in acetate, formate, ethanol and succinate production as major products (Fig. 5). Therefore, we considered to study the genes experimentally which play key roles in influencing these by-product formation pathways. And other genes were rejected on the basis of their performance in *in-silico* studies, and their relative effect on the biohydrogen production in *E. coli*, to support better growth and production rates and save time. The yields obtained by single gene mutations are low relative to theoretical yields, but laid the foundation for our further studies.

3.4. Extracellular metabolite profiling

Residual sugars and by-products synthesised by the cell corresponds to the intracellular changes resultant to the perturbations done in the genome. Sugar preferences of *E. coli* when grown in the mixture of glucose, xylose and arabinose, exhibit the preferences consuming glucose first followed by the arabinose and xylose at last [26]. The mixture of sugars can induce the carbon catabolite repression (CCR) mediated through the intricate balances of key components carbohydrate specific enzyme II complex domain A bound to glucose ($EIIA^{Glc}$), cAMP receptor protein (CRP), cAMP (signal metabolite) and adenylate cyclase (AC) [80]. The phosphorylation status of $EIIA^{Glc}$ and availability of phosphoenolpyruvate as a phosphate donor influences the glucose metabolism. The primary sugar metabolized is glucose and is phosphorylated readily resulting in the non-phosphorylated form of $EIIA^{Glc}$ which acts as a stimulus for low cAMP pools. Unavailability of P-$EIIA^{Glc}$ induces the catabolite repression for non-PTS (phosphotransferase system) sugars. Also, depletion of glucose and availability of P-$EIIA^{Glc}$ increases the intracellular levels of cAMP as an activation of AC. The elevated cAMP forms cAMP-Crp complex which activates the genes responsible for metabolising the arabinose and xylose sugars [81]. The cognate sugar and transcription factor complex with cAMP-CRP complex regulate the transport of respective sugar and its metabolic genes. Among arabinose and xylose, arabinose is metabolized first and AraC-arabinose complex binds to the xylose promoters inhibiting its transcription inducing the CCR [81]. The CCR effect resulted in the diauxic growth which was evident in our study as well when grown on mixed sugars (Supplementary Fig. S2). Also following discussion states the consumption of these sugar substrates and production of metabolites after dark fermentation.

WT and mutants grown in mixed sugars or only glucose as a substrate, they reflect the same sugar preferences; and ethanol being the most dominated product of fermentation followed by formate and succinate. It was observed that after 24-h incubation, 2.9 mM (*ΔhyaB)* of glucose was unutilised by the cell when grown in mixed sugars as a substrate. This could be due to maximum glucose was utilised for the cell biomass generation in logarithmic phase and later the secondary sugars take over showing diauxic growth (Supplementary Fig. S2) and hydrogen production stage, as evident. Essentially anaerobic and acidic conditions are primary drivers of the hydrogen production and thus arabinose was the secondary sugar completely utilised by the *E. coli*. Metabolite profile of WT shows almost complete consumption of the glucose and arabinose, while leaving xylose maximum at 60 % utilization by WT and least by *ΔfrdC* (8.3788 % utilised). Whereas, only *ΔhyaB* showed residual glucose of 2.923 mM also attributing to its slow growth as evident from its growth data. The *ΔfrdC* was assessed to show the highest formate production of 12.701 mM, which was transported extracellularly (Fig. 5b).

Elevated formate concentration in *ΔfrdC* suggests its synergistic role in hydrogen production for further studies. Moreover multiple studies report the *frdC* gene knockout, in order to increase the hydrogen productivity and this study align with the same [82]. Though *frdC* gene was disrupted, succinate was evidently produced in significant quantities which implies the involvement of reductive TCA branch which operates through malate intermediates for succinate production. As per earlier studies done for hydrogen production and growth kinetics of *E. coli* evidently states that the defect in hydrogenases affects the growth and hydrogen productivity [68]. In this work, it was reflected the same, regarding the hydrogen productivity and its effect on the reduced formate production of 3.1976 mM and 3.2762 mM of formate when *ΔhyaB* and *ΔhybC* were disrupted, respectively (Fig. 5b). As this secreted formate is reuptaken by the cell and converted to hydrogen gas by the FHL complex. Whereas, hydrogenase-3 and -4 are reported to be hydrogen evolving hydrogenases, this study has only considered studying two uptake hydrogenases namely Hyd-1 and -2. Ethanol being persistent in all the mutants and its synthesis is biochemically important to maintain the redox-balance in the cell, its synthesis is inevitable. The mutants of *ΔhyaB* and *ΔackA* affected the ethanol production. Although the glucose was utilised for the biomass production in log phase, decreased pH and pentoses result in the by-products which states the dynamic behaviour of mutants adapting to attain the cellular homeostasis. Similarly, when only glucose was used as a substrate sugar for the hydrogen production, overall product portfolio remained the same consisting of ethanol, succinate and formate. Also, we found the acetic acid and lactic acid production was negligible. Glucose as a sole sugar substrate, it was maximally consumed by the WT (89 %) followed by *ΔadhE* (77.5 %), while least was consumed by *ΔhybC* (54.22 %) (Fig. 5a). Surprisingly, *ΔhyaB* showed the least amount of ethanol produced amongst all the mutants with 0.65 mM ethanol while *ΔhybC* produced 14.21 mM and was highest. As the product portfolio is dominated by ethanol and succinate, further hydrogen improving strategies were designed to minimize concentration of these products to increase the flux towards formate synthesis. This is the first study where we

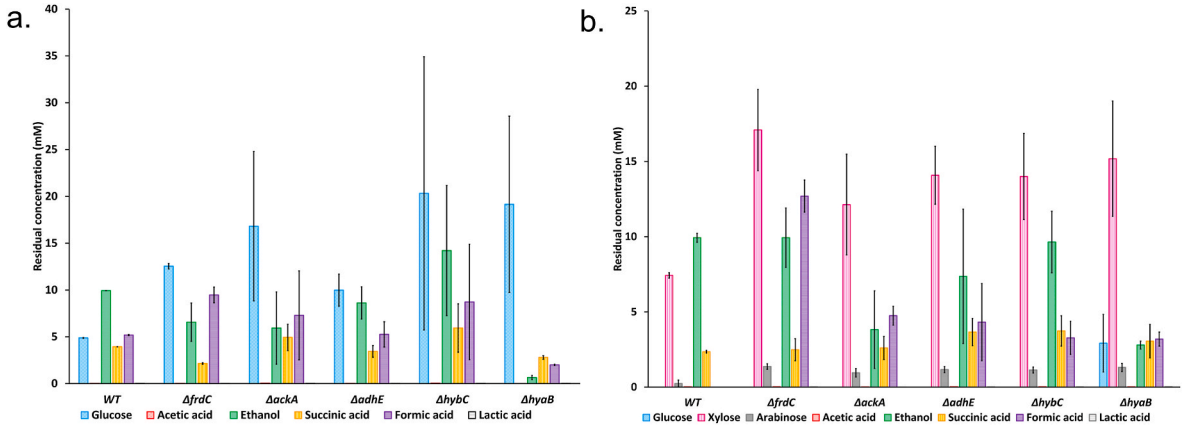

Fig. 5. Metabolic profiling of mutant-grown media filtrate after fermentation (a) Glucose as a substrate and, (b) Mixed sugar as a substrate.

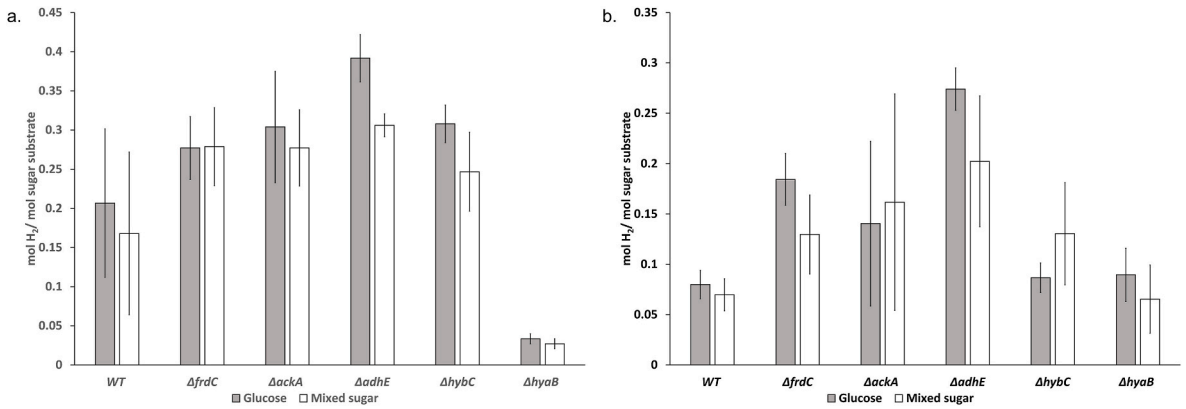

Fig. 6. Hydrogen yields of mutants grown in glucose or mixed sugars as a carbon substrate. (a) Yields at 30 °C and, (b) Yields at 37 °C.

demonstrated the bH_2 production and simultaneously assessing the formate accumulation with the sugar usage, providing the clear understanding of each gene involvement in bH_2 production physiology in the cell.

Hydrogen production in the cell is triggered as a result of the maintaining the energy balance and as a response to pH and anaerobic conditions [83]. It is a native mechanism to counteract the dynamic niche of the cell in absence of oxygen where the medium is acidified. Thus, to exploit this pathway of hydrogen production known as pyruvate-formate lyase (PFL) pathway, it is activated by the cell for maintaining the reducing equivalents. Anaerobic mixed-fermentation in *E. coli* results in the production of various acids and alcohols depending on the environmental conditions and genetic constitution. Understanding independent role of each perturbation in genome facilitates to understand the behaviour of cell in the niche and helps in framing a strategy to develop a robust engineered strain for production.

This study infers that; genetic and biochemical wiring is intricately designed for cell to adapt to changes made within and around. Sophisticated GEMs are certainly agreeable to predict the outcome of perturbations made in the genome and its environment. This study paved the platform how to explore the GEM to the full potential to assess the cellular system while targeting genes for the metabolic engineering. This study justifies the use of GEMs for developing a strategy and optimizing it for hydrogen production with the said set of rules that abides model to perform in optima. The cell is a dynamic entity and corresponds to a miniscule change in its environment, it would be convenient to improve these models which can perform the predictions for mixture of substrates used to replicate situations such as lignocellulosic sugar mixtures (glucose, xylose and arabinose). Such studies will help GEMs to be developed further for more accurate analysis and predictions. There are few genes such as *narL* and *hycA* which are not present in the GEM and certainly limit its accuracy of predictions as studies prove their involvement in influencing the hydrogen production in *E. coli* [14]. Also, we were unable to study their independent role on influencing hydrogen production as we did for genes enlisted in Table 1. Even though simulations were gullible, GEMs still need to be developed further for accuracy as the data becomes available. As we explored only Parsimonious enzyme usage flux balance analysis (pFBA) and minimization of metabolic adjustments (MOMA) methods for predicting the effects of perturbation in this study, we also encourage further analysis using a different methods available such as regulatory on-off minimization (ROOM) to study optimum conditions based of the product portfolio selection in near future [84]. Experimental data based on the predictions suffices for the GEMs application in the bH_2 production and biomass accumulation which proves to be a powerful tool to be harnessed in the field of biotechnology. Cell biomass of perturbed systems comparable to WT strain, is a desirable trait for industrial application and we have analysed effect of single gene deletions in MG1655 strain of *E. coli* which corroborates with the previous studies of hampered growth as a result of genome reduction affecting the cellular growth and stability. Thus, this study illustrates the application of CRISPR-Cas9 technology in disrupting the gene with minimal genome reduction (Supplementary Fig. S3). Hence, individual effect on the cellular growth where gene perturbations such as *ΔhybC*, *ΔackA*, *ΔadhE* and *ΔfrdC* show at par or better growth rates than WT, except *ΔhyaB*, demonstrating a successful implementation of this modulation technology for metabolic engineering in view of growth studies [74]. As growth is an important aspect of this study, we demonstrated its effect on bH_2 production establishing direct relation of a biomass coupled to better product assimilation profiles.

Although our study considers the synthetic mixture of lignocellulosic fermentable sugars, there are more challenges while using the hydrolysed lignocellulose biomass [29]. Complex structure of lignocellulose after breaking down usually using physico-chemical and enzymatic methods result in inhibitory by-products such as furfurals, 5-hydroxymethylfurfural (HMF) and acids that interfere with microbial growth (Table 3) [29,85]. These challenges still persist and their effects were neither predicted by GEM nor we could demonstrate it in our experimental setup. This study only demonstrated the application of GEM in the field of bH_2 production in *E. coli* MG1655 and does not advocate for the limitations of using actual lignocellulosic sugars which is beyond the scope of this study and stand as a knowledge gap for the future studies. This study provides the foundation for future development of metabolically engineered strains involving *in-silico* guided strategies to achieve the optimized industrial strain (work in progress). Our work supports as a foundation upon which further strategy was framed for increasing bH_2 production.

4. Conclusion

In this study, we successfully demonstrated the application of GEM *i*ML1515 of *E. coli* MG1655 strain in predicting the effect of gene perturbations on cell growth and formate production which is directly proportional to hydrogen production. It is the first study of this type integrating the potential of GEM to the optimal production of bH_2 keeping the cell biomass conserved without perturbing the growth rates and cell biomass generation. This advanced tool was able to simulate the complex and dynamic cellular behaviour of *E. coli* cell and predict the outcome based on the substrate sugar in anaerobic conditions. Also, we experimentally proved the same by perturbing genes *hyaB*, *hybC*, *frdC*, *ackA* and *adhE* to show the credibility of *in-silico* outcomes aligning these results. *In-silico* and experimental results showed formate and hydrogen production, respectively corresponding to each other establishing direct relation. Also, it was able to reflect the *in-silico* results for lignocellulosic mixed sugar, proving its application for future in predicting waste substrate resources that can be used as carbon feedstock for cellular biomass

cultivation with sustenance. Here, we also show the relation of increased growth rate coupled to better hydrogen production (*adhE*) and effect of temperature on the production of hydrogen. These results are promising to ease the process of designing strategies for growth coupled product formation in the area of metabolic engineering. This study can pave way for the holistic approach towards scaleup production of bH_2 with uncompromised biomass growth rate. Thus, optimal productivity of bH_2 which could stand at the commercial potential adopting waste biomass derived sugars such as lignocellulosic sugars as considered in this study.

CRediT authorship contribution statement

Tanushree Baldeo Madavi: Writing – original draft, Visualization, Software, Formal analysis, Data curation. **Vini Madathil:** Writing – review & editing, Resources, Project administration, Data curation. **V.M. Aishwarya:** Resources, Data curation. **Kwon-Young Choi:** Supervision, Conceptualization. **Sushma Chauhan:** Writing – review & editing, Supervision, Investigation, Conceptualization. **Sudheer D.V. N. Pamidimarri:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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References

[1] Gundimeda H, Köhlin G. Fuel demand elasticities for energy and environmental policies: indian sample survey evidence. Energy Econ 2008;30(2):517–46.
[2] Wilkinson P, Smith KR, Joffe M, Haines A. A global perspective on energy: health effects and injustices. Lancet 2007;370(9591):965–78.
[3] Höök M, Tang X. Depletion of fossil fuels and anthropogenic climate change—A review. Energy Policy 2013;52:797–809.
[4] Liu Y, Cruz-Morales P, Zargar A, Belcher MS, Pang B, Englund E, Dan Q, Yin K, Keasling JD. Biofuels for a sustainable future. Cell 2021;184(6):1636–47.
[5] Johnsson F, Jan K, Rootzén J. The threat to climate change mitigation posed by the abundance of fossil fuels. Clim Policy 2019;19(2):258–74.
[6] Wuebbles DJ, Jain AK. Concerns about climate change and the role of fossil fuel use. Fuel Process Technol 2001;71(1):99–119.
[7] Show KY, Lee DJ, Tay JH, Lin CY, Chang JS. Biohydrogen production: current perspectives and the way forward. Int J Hydrogen Energy 2012;37(20):15616–31.
[8] Abe JO, Popoola API, Ajenifuja E, Popoola OM. Hydrogen energy, economy and storage: review and recommendation. Int J Hydrogen Energy 2019;44(29):15072–86.
[9] Pal DB, Singh A, Bhatnagar A. A review on biomass based hydrogen production technologies. Int J Hydrogen Energy 2022;47(3):1461–80.
[10] Xu X, Zhou Q, Yu D. The future of hydrogen energy: bio-hydrogen production technology. Int J Hydrogen Energy 2022;47(79):33677–98.
[11] Madavi TB, Chauhan S, Keshri A, Alavilli H, Choi K-Y, Pamidimarri SDVN. Whole-cell biocatalysis: advancements toward the biosynthesis of fuels. Biofuel Bioprod Biorefining 2022;16(3):859–76.
[12] Madavi TB, Chauhan S, Jha M, Choi K-Y, Pamidimarri SD. Biohydrogen machinery: recent insights, genetic fabrication, and future prospects. Chem Eng Technol 2023;46(2):179–90.
[13] Madavi TB, Hariharno S, Singh A, Chauhan S, Pamidimarri SD. Biofuels: fueling the future with whole-cell-derived fuels, whole-cell biocatalysis. Apple Academic Press; 2024. p. 309–34.
[14] Fan Z, Yuan L, Chatterjee R. Increased hydrogen production by genetic engineering of Escherichia coli. PLoS One 2009;4(2):e4432.
[15] F. Sargent, Chapter eight - the model [NiFe]-Hydrogenases of Escherichia coli, in: R.K. Poole (Ed.), Advances in microbial physiology, Academic Press2016, pp. 433-507.
[16] Bagramyan K, Trchounian A. Structural and functional features of formate hydrogen lyase, an enzyme of mixed-acid fermentation from Escherichia coli. Biochemistry (Mosc) 2003;68(11):1159–70.
[17] Maeda T, Sanchez-Torres V, Wood TK. Hydrogen production by recombinant Escherichia coli strains. Microb Biotechnol 2012;5(2):214–25.
[18] Clark DP. The fermentation pathways of Escherichia coli. FEMS Microbiol Rev 1989;5(3):223–34.
[19] Yoshida A, Nishimura T, Kawaguchi H, Inui M, Yukawa H. Enhanced hydrogen production from glucose using ldh- and frd-inactivated Escherichia coli strains. Appl Microbiol Biotechnol 2006;73(1):67–72.
[20] Yoshida A, Nishimura T, Kawaguchi H, Inui M, Yukawa H. Enhanced hydrogen production from formic acid by formate hydrogen lyase-overexpressing Escherichia coli strains. Appl Environ Microbiol 2005;71(11):6762–8.
[21] Hu H, Wood TK. An evolved Escherichia coli strain for producing hydrogen and ethanol from glycerol. Biochem Biophys Res Commun 2010;391(1):1033–8.
[22] Singh A, Sevda S, Abu Reesh IM, Vanbroekhoven K, Rathore D, Pant D. Biohydrogen production from lignocellulosic biomass: technology and sustainability. Energies 2015;8(11):13062–80.
[23] Lopez-Hidalgo AM, Sánchez A, De León-Rodríguez A. Simultaneous production of bioethanol and biohydrogen by Escherichia coli WDHL using wheat straw hydrolysate as substrate. Fuel 2017;188:19–27.
[24] Taifor AF, Zakaria MR, Mohd Yusoff MZ, Toshinari M, Hassan MA, Shirai Y. Elucidating substrate utilization in biohydrogen production from palm oil mill effluent by Escherichia coli. Int J Hydrogen Energy 2017;42(9):5812–9.
[25] Kurokawa M, Seno S, Matsuda H, Ying B-W. Correlation between genome reduction and bacterial growth. DNA Res 2016;23(6):517–25.
[26] Wu Y, Shen X, Yuan Q, Yan Y. Metabolic engineering strategies for Co-Utilization of carbon sources in microbes. Bioengineering 2016;3(1):10.
[27] Taifor AF, Zakaria MR, Yusoff MZM, Toshinari M, Hassan MA, Shirai Y. Elucidating substrate utilization in biohydrogen production from palm oil mill effluent by Escherichia coli. Int J Hydrogen Energy 2017;42(9):5812–9.
[28] Poladyan A, Baghdasaryan L, Trchounian A. Escherichia coli wild type and hydrogenase mutant cells growth and hydrogen production upon xylose and glycerol co-fermentation in media with different buffer capacities. Int J Hydrogen Energy 2018;43(33):15870–9.
[29] Sivagurunathan P, Kumar G, Mudhoo A, Rene ER, Saratale GD, Kobayashi T, Xu K, Kim S-H, Kim D-H. Fermentative hydrogen production using lignocellulose biomass: an overview of pre-treatment methods, inhibitor effects and detoxification experiences. Renew Sustain Energy Rev 2017;77:28–42.
[30] Clark DP. The fermentation pathways of Escherichia coli. FEMS Microbiol Rev 1989;5(3):223–34.
[31] Petersen J-E. Energy production with agricultural biomass: environmental implications and analytical challenges†. Eur Rev Agric Econ 2008;35(3):385–408.
[32] Saini JK, Saini R, Tewari L. Lignocellulosic agriculture wastes as biomass feedstocks for second-generation bioethanol production: concepts and recent developments. 3 Biotech 2015;5(4):337–53.
[33] Phan PT, Nguyen B-S, Nguyen T-A, Kumar A, Nguyen V-H. Lignocellulose-derived monosugars: a review of biomass pre-treating techniques and post-methods to produce sustainable biohydrogen. Biomass Conversion and Biorefinery 2023;13(10):8425–39.
[34] Basak B, Jeon B-H, Kim TH, Lee J-C, Chatterjee PK, Lim H. Dark fermentative hydrogen production from pretreated lignocellulosic biomass: effects of inhibitory byproducts and recent trends in mitigation strategies. Renew Sustain Energy Rev 2020;133:110338.
[35] Haroun BM, Nakhla G, Hafez H, Nasr FA. Impact of furfural on biohydrogen production from glucose and xylose in continuous-flow systems. Renew Energy 2016;93:302–11.
[36] Akobi C, Hafez H, Nakhla G. The impact of furfural concentrations and substrate-to-biomass ratios on biological hydrogen production from synthetic lignocellulosic hydrolysate using mesophilic anaerobic digester sludge. Bioresour Technol 2016;221:598–606.
[37] Nielsen J, Keasling JD. Engineering cellular metabolism. Cell 2016;164(6):1185–97.
[38] Pereira F, Lopes H, Maia P, Meyer B, Nocon J, Jouhten P, Konstantinidis D, Kafkia E, Rocha M, Kötter P, Rocha I, Patil KR. Model-guided development of an evolutionarily stable yeast chassis. Mol Syst Biol 2021;17(7):e10253.
[39] Jian X, Li N, Chen Q, Hua Q. Model-guided identification of novel gene amplification targets for improving succinate production in Escherichia coli NZN111. Integr Biol : quantitative biosciences from nano to macro 2017;9(10):830–5.
[40] Chinnasamy Perumal R, Selvaraj A, Ramesh Kumar G. Elementary flux mode analysis of acetyl-CoA pathway in Carboxydothermus hydrogenoformans Z-2901. Advances in Bioinformatics 2014;2014(1):928038.
[41] Cheng H-H, Whang L-M, Lin C-A, Liu I-C, Wu C-W. Metabolic flux network analysis of fermentative hydrogen production: using Clostridium tyrobutyricum as an example. Bioresour Technol 2013;141:233–9.
[42] Manish S, Venkatesh K, Banerjee R. Metabolic flux analysis of biological hydrogen production by Escherichia coli. Int J Hydrogen Energy 2007;32(16):3820–30.
[43] Monk JM, Lloyd CJ, Brunk E, Mih N, Sastry A, King Z, Takeuchi R, Nomura W, Zhang Z, Mori H. I ML1515, a knowledgebase that computes Escherichia coli traits. Nat Biotechnol 2017;35(10):904–8.

[44] Gopalakrishnan S, Maranas CD. 13C metabolic flux analysis at a genome-scale. Metab Eng 2015;32:12–22.
[45] Oberhardt MA, Palsson BØ, Papin JA. Applications of genome-scale metabolic reconstructions. Mol Syst Biol 2009;5(1):320.
[46] Rocha I, Maia P, Evangelista P, Vilaça P, Soares S, Pinto JP, Nielsen J, Patil KR, Ferreira EC, Rocha M. OptFlux: an open-source software platform for in silico metabolic engineering. BMC Syst Biol 2010;4:1–12.
[47] Horvath P, Barrangou R. CRISPR/Cas, the immune system of bacteria and archaea. Science 2010;327(5962):167–70.
[48] Hu LF, Li YX, Wang JZ, Zhao YT, Wang Y. Controlling CRISPR-Cas9 by guide RNA engineering. Wiley Interdisciplinary Reviews: RNA 2023;14(1):e1731.
[49] Zhao D, Zhu X, Zhou H, Sun N, Wang T, Bi C, Zhang X. CRISPR-based metabolic pathway engineering. Metab Eng 2021;63:148–59.
[50] Ko Y-S, Kim JW, Lee JA, Han T, Kim GB, Park JE, Lee SY. Tools and strategies of systems metabolic engineering for the development of microbial cell factories for chemical production. Chem Soc Rev 2020;49(14):4615–36.
[51] Oesterle S, Wuethrich I, Panke S. Toward genome-based metabolic engineering in bacteria. Adv Appl Microbiol 2017;101:49–82.
[52] Blin K, Pedersen LE, Weber T, Lee SY. CRISPy-web: an online resource to design sgRNAs for CRISPR applications. Synthetic and Systems Biotechnology 2016;1(2):118–21.
[53] Maeda T, Sanchez-Torres V, Wood TK. Hydrogen production by recombinant Escherichia coli strains. Microb Biotechnol 2012;5(2):214–25.
[54] King ZA, Lu J, Dräger A, Miller P, Federowicz S, Lerman JA, Ebrahim A, Palsson BO, Lewis NE. BiGG models: a platform for integrating, standardizing and sharing genome-scale models. Nucleic Acids Res 2015;44(D1):D515–22.
[55] Maeda T, Sanchez-Torres V, Wood TK. Metabolic engineering to enhance bacterial hydrogen production. Microb Biotechnol 2008;1(1):30–9.
[56] Beyer L, Doberenz C, Falke D, Hunger D, Suppmann B, Sawers RG. Coordination of FocA and pyruvate formate-lyase synthesis in Escherichia coli demonstrates preferential translocation of formate over other mixed-acid fermentation products. J Bacteriol 2013;195(7):1428–35.
[57] Gupta S, Clark DP. Escherichia coli derivatives lacking both alcohol dehydrogenase and phosphotransacetylase grow anaerobically by lactate fermentation. J Bacteriol 1989;171(7):3650–5.
[58] Cunningham PR, Clark DP. The use of suicide substrates to select mutants of Escherichia coli lacking enzymes of alcohol fermentation. Mol Gen Genet MGG 1986;205(3):487–93.
[59] Alam KY, Clark DP. Anaerobic fermentation balance of Escherichia coli as observed by in vivo nuclear magnetic resonance spectroscopy. J Bacteriol 1989;171(11):6213–7.
[60] Maeda T, Sanchez-Torres V, Wood TK. Enhanced hydrogen production from glucose by metabolically engineered Escherichia coli. Appl Microbiol Biotechnol 2007;77(4):879–90.
[61] Valle A, Cantero D, Bolívar J. Metabolic engineering for the optimization of hydrogen production in Escherichia coli: a review. Biotechnol Adv 2019;37(5):616–33.
[62] Maeda T, Tran KT, Yamasaki R, Wood TK. Current state and perspectives in hydrogen production by Escherichia coli: roles of hydrogenases in glucose or glycerol metabolism. Appl Microbiol Biotechnol 2018;102:2041–50.
[63] Maeda T, Sanchez-Torres V, Wood TK. Enhanced hydrogen production from glucose by metabolically engineered Escherichia coli. Appl Microbiol Biotechnol 2007;77:879–90.
[64] Maeda T, Sanchez-Torres V, Wood TK. Escherichia coli hydrogenase 3 is a reversible enzyme possessing hydrogen uptake and synthesis activities. Appl Microbiol Biotechnol 2007;76:1035–42.
[65] Maeda T, Sanchez-Torres V, Wood TK. Metabolic engineering to enhance bacterial hydrogen production. Microb Biotechnol 2008;1(1):30–9.
[66] Wang J, Wan W. Optimization of fermentative hydrogen production process by response surface methodology. Int J Hydrogen Energy 2008;33(23):6976–84.
[67] Jiang Y, Chen B, Duan C, Sun B, Yang J, Yang S. Multigene editing in the Escherichia coli genome via the CRISPR-Cas9 system. Appl Environ Microbiol 2015;81(7):2506–14.
[68] Poladyan A, Trchounian A. Characterization of hydrogen production by Escherichia coli wild-type and mutants of hydrogenases utilizing Xylose as fermentation substrate. BioEnergy Research 2019;12(4):1033–41.
[69] Mathews J, Li Q, Wang G. Characterization of hydrogen production by engineered Escherichia coli strains using rich defined media. Biotechnol Bioproc Eng 2010;15(4):686–95.
[70] Wang Z-H, Tan J-Y, Zhang Y-T, Ren N-Q, Zhao L. Evaluating bio-hydrogen production potential and energy conversion efficiency from glucose and xylose under diverse concentrations. Fermentation 2022;8(12):739.
[71] Poladyan A, Avagyan A, Vassilian A, Trchounian A. Oxidative and reductive routes of glycerol and glucose fermentation by Escherichia coli batch cultures and their regulation by oxidizing and reducing reagents at different pHs. Curr Microbiol 2013;66(1):49–55.
[72] Madavi TB, Chauhan S, Jha M, Choi K-Y, Pamidimarri SDVN. Biohydrogen machinery: recent insights, genetic fabrication, and future prospects. Chem Eng Technol 2023;46(2):179–90.
[73] Yoshida A, Nishimura T, Kawaguchi H, Inui M, Yukawa H. Enhanced hydrogen production from glucose using ldh-and frd-inactivated Escherichia coli strains. Appl Microbiol Biotechnol 2006;73:67–72.
[74] Maeda T, Sanchez-Torres V, Wood TK. Metabolic engineering to enhance bacterial hydrogen production. Microb Biotechnol 2008;1(1):30–9.
[75] Yadav M, Paritosh K, Vivekanand V. Lignocellulose to bio-hydrogen: an overview on recent developments. Int J Hydrogen Energy 2020;45(36):18195–210.
[76] Gadgil M, Kapur V, Hu WS. Transcriptional response of Escherichia coli to temperature shift. Biotechnol Prog 2005;21(3):689–99.
[77] Jones PG, VanBogelen RA, Neidhardt FC. Induction of proteins in response to low temperature in Escherichia coli. J Bacteriol 1987;169(5):2092–5.
[78] Farewell A, Neidhardt FC. Effect of temperature on in vivo protein synthetic capacity in Escherichia coli. J Bacteriol 1998;180(17):4704–10.
[79] Marr AG. Growth rate of Escherichia coli. Microbiol Rev 1991;55(2):316–33.
[80] Gabor E, Göhler A-K, Kosfeld A, Staab A, Kremling A, Jahreis K. The phosphoenolpyruvate-dependent glucose–phosphotransferase system from Escherichia coli K-12 as the center of a network regulating carbohydrate flux in the cell. EJCB (Eur J Cell Biol) 2011;90(9):711–20.
[81] Balderas-Hernandez VE, Landeros Maldonado KP, Sánchez A, Smoliński A, De Leon Rodriguez A. Improvement of hydrogen production by metabolic engineering of Escherichia coli: modification on both the PTS system and central carbon metabolism. Int J Hydrogen Energy 2020;45(9):5687–96.
[82] Valle A, Cabrera G, Muhamadali H, Trivedi DK, Ratray NJW, Goodacre R, Cantero D, Bolivar J. A systematic analysis of TCA Escherichia coli mutants reveals suitable genetic backgrounds for enhanced hydrogen and ethanol production using glycerol as main carbon source. Biotechnol J 2015;10(11):1750–61.
[83] Zinoni F, Beier A, Pecher A, Wirth R, Böck A. Regulation of the synthesis of hydrogenase (formate hydrogen-lyase linked) of E. coli. Arch Microbiol 1984;139(4):299–304.
[84] Lewis NE, Hixson KK, Conrad TM, Lerman JA, Charusanti P, Polpitiya AD, Adkins JN, Schramm G, Purvine SO, Lopez-Ferrer D, Weitz KK, Eils R, König R, Smith RD, Palsson BØ. Omic data from evolved *E. coli* are consistent with computed optimal growth from genome-scale models. Mol Syst Biol 2010;6(1):390.
[85] Cao G-L, Ren N-Q, Wang A-J, Guo W-Q, Xu J-F, Liu B-F. Effect of lignocellulose-derived inhibitors on growth and hydrogen production by Thermoanaerobacterium thermosaccharolyticum W16. Int J Hydrogen Energy 2010;35(24):13475–80.
[86] Chen W-H, Lin T-S, Guo G-L, Huang W-S. Ethanol production from rice straw hydrolysates by Pichia stipitis. Energy Proc 2012;14:1261–6.
[87] da Conceição Gomes A, Rodrigues MI, de França Passos D, Machado de Castro A, Maria Mello Santa Anna L, Pereira N. Acetone–butanol–ethanol fermentation from sugarcane bagasse hydrolysates: utilization of C5 and C6 sugars. Electron J Biotechnol 2019;42:16–22.
[88] van Dijk M, Mierke F, Nygård Y, Olsson L. Nutrient-supplemented propagation of Saccharomyces cerevisiae improves its lignocellulose fermentation ability. AMB Express 2020;10(1):157.