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Elementary flux mode analysis of central carbon metabolism in *Caldicellulosiruptor saccharolyticus* to enhance hydrogen production

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***Abstract*—** Microbial hydrogen production from biomass has been perceived as an imperative wellspring of renewable energy. *Caldicellulosiruptor saccharolyticus* has proven itself to be an excellent candidate for biological hydrogen production. Hydrogen is produced as a biological product from central carbon metabolism pathway by the utilization of various soluble sugar substrates. An organism's metabolism is a key factor in understanding its physiology, therefore, metabolic network of central carbon metabolism of *C. saccharolyticus* was reconstructed. The reconstructed and refined central carbon metabolism network comprises 31 reactions (excluding the efflux and influx reactions) and 40 metabolites in total. An elementary flux mode analysis of central carbon metabolism was carried out to investigate the underlying biochemical mechanism of hydrogen production by determining their intermediate fluxes, using linear programming (LP) method available in CellNetAnalyzer software. An *in silico* gene knockout of lactate dehydrogenase (*ldh*) and pyruvate kinase (*pyk*) of the modeled central carbon metabolism pathway allows the maximum theoretical hydrogen yield of 46.2758 mmol/gDW/hr. The single gene knockout of *ldh* gene tends to increase the flux rate of hydrogen to 32%. Similarly, double gene knockout of *ldh* and *pyk* genes yielded an increase in hydrogen flux rate to 35%. Ultimately, this elementary flux mode analysis study of central carbon metabolism pathway of *C. saccharolyticus* with glucose as substrate or carbon source would further act as a model for strain improvement.

Keywords— *Caldicellulosiruptor saccharolyticus; Biohydrogen; pathway reconstruction; elementary modes.*

I. INTRODUCTION

Extensive utilization of fossil fuels to achieve the world's energy demands is responsible for changes in the worldwide atmosphere with an emission of pollutants. However, fossil fuels are confined and unevenly distributed around the globe [1,2]. Recent reviews on hydrogen production indicated that a group of conceivable fuel sources is being examined and renewable energy resources are proposed as an option, which is fit for overcoming the issues on environmental problems. As hydrogen [H_2] is non-polluting in nature and smolders to form water which can be further reused, it is believed to be the most promising future fuel [3,4]. H_2 gas is not only used as a wellspring of energy but is also extensively used for the creation of chemicals, for the hydrogenation of fats, in the nourishment business for margarine creation and processing steel [5,6]. BioHydrogen is considered as the cleanest fuel and makes utilization of microorganisms that tend to produce H_2 from lignocellulosic biomass and waste materials [7,8,9,10,11]. For the past many years, a lot of work has been carried out for improving Biohydrogen yield which is one of the major bottleneck [6]. However, for further improvement, a good understanding of the metabolic network is of considerable importance.

To analyze metabolic networks, theoretical and computational methods have been proved useful [12,13,14,15]. Elementary flux mode (EFM) analysis is one of the powerful tools for metabolic pathway analysis [16,17]. EFM allows the detection of all potential routes for synthesizing certain products and hence enables us to calculate all possible steady-state flux distributions of the network, thereby determining the theoretical molar yield of products and studying their effects on any genetic modifications [18,19,20]. Elementary mode studies have been used for polyhydroxybutanoate production from *Saccharomyces cerevisiae* [21,22,23] and to develop a rational model of methionine production from organisms such as *Escherichia coli* and *Corynebacterium glutamicum* [24]. Recently, elementary flux mode analysis has also been used for penicillin synthesis in *Penicillium chrysogenum* [15]. Study of H_2 production in wild-type *E. coli* using experimental analysis resulted in a flux distribution indicating a H_2 production of 0.17 mol per mole of glucose consumption [25]. H_2 production of *E. coli* through

metabolic flux analysis with *ldh* gene deletion was predicted to be 0.23 mol per mole of glucose consumed which is slightly higher than the wild-type strain. In another study based on computational flux analysis, H_2 production in *E. coli* via formate hydrogen lyase reaction suggested that the level of hydrogen production matches experimental observations [26].

Caldicellulosiruptor saccharolyticus which was isolated from the Rotorua-Taupa thermal area in New Zealand is an extremely thermophilic, strictly anaerobic asporogenous bacterium, that exhibits various interesting properties like high H_2 yield (maximum 3.3 to 3.6 mol H_2 per mol glucose), insensitivity to high P_{H2} and ability to hydrolyse a variety of polymeric carbohydrates. It can grow at a temperature range of 45-80 °C (Topt=70 °C) and pH range of 5.5-8.0 (pHopt=7) [27,28]. A thorough understanding of central carbon metabolism of *C. saccharolyticus* provides a basic model for the characterization of H_2 production mechanism. The main pathways involved in central carbon metabolism are Embden-Meyerhof pathway, non-oxidative pentose phosphate pathway, pyruvate pathway, malate shunt and citrate cycle pathway.

In the present study, an elementary mode flux balance analysis study has been carried out for the newly reconstructed central carbon metabolism pathway of *C. saccharolyticus* that comprises of reaction stoichiometry information. *In silico* gene knockouts studies were carried out for the central carbon metabolism pathway and the theoretical molar yield for H_2 was calculated. By using the concept of elementary modes we hereby provide a basis to design a system that has specific phenotypes, metabolic network regulation and robustness that expedite the application of metabolic engineering strategies to enhance the hydrogen productivity of *C. saccharolyticus.*

II. MATERIALS AND METHODS

Metabolic pathway reconstruction

In the first stage, a draft model for central carbon metabolism was built using biochemical information from online databases like KEGG, BioCyc and BRENDA, which provide comprehensive information on enzymes and biochemical reactions. A network was created using genes, proteins and metabolites involved in the metabolism of the organism of interest. Manual curation is another important step in metabolic network reconstruction process which involves re-evaluation and refinement of the draft reconstruction [29]. The draft network might contain dead ends or some missing reactions known as gaps that links the compounds or metabolites with the rest of the pathway. Therefore, refinement of the network through gap analysis becomes mandatory [30,31,32]. Gap filling was achieved by adding organism-specific information from the manually re-annotated results [28]. If no organism-specific information was available, information for phylogenetically close organisms was used and marked as such. Reactions containing generic terms, such as protein, DNA, electron acceptor, etc. were not included as they normally serve as space holders in databases. Protons and water were added to the reactions to balance the elements on both sides as some databases and many biochemical textbooks omit these molecules.

Elementary flux mode analysis

Steady-state metabolic flux vectors with a minimal set of active reactions are known as elementary modes (EMs). EMs study is an important step for analyzing the production of biotechnologically important metabolites [33]. The METATOOL [34] program available in CellNetAnalyzer [35] was compiled using MATLAB environment to generate EMs. The objectives of the reactions that maximize the flux rate of products were calculated using Linear Programming (LP). The theoretical fluxes were calculated using CPLEX solver in CellNetAnalyzer for products such as H_2, acetate and lactate. In the present study, our objective is to increase the flux towards H_2. The theoretical molar yield of any product is represented by [*vp/vs*], where *vp* is the flux rate of product and *vs* is the flux rate of substrate. The flux balance analysis is represented by the following Equation:

$$\sum_{j} S_{ij}\, v_j = 0 \qquad \forall i \in M_i \tag{1}$$

where S_{ij} is the stoichiometric coefficient of the i^{th} metabolite in the j^{th} reaction, V_j is the flux of the j^{th} reaction and M_i is the internal metabolite.

Gene knockout using the CellNetAnalyzer tool

CellNetAnalyzer simulations were run to complete for single and double gene knockout. The knockout genes were lactate dehydrogenase (*ldh*) and pyruvate kinase (*pyk*). Using glucose as a substrate to increase H_2 production, a gene(s) knockout study was performed in *C. saccharolyticus* stoichiometric model.

III. RESULTS

Reconstruction and refinement of Central Carbon Metabolism

A detailed comprehension of central carbon metabolism of *C. saccharolyticus* provides a basic model for the characterization of H_2 production mechanism. The draft reconstructed model of central carbon metabolism of *C. saccharolyticus* (Figure 1) showed 45 genes catalyzing 28 reactions among which 12 reactions are irreversible and 16 are reversible. Pathway gaps were found in the central carbon metabolism as reported in the KEGG pathway database and were filled by analyzing the missing genes. As

a result, a reaction catalyzed by the enzyme fumarate reductase/succinate dehydrogenase flavoprotein domain protein (EC: 1.3.5.1/1.3.5.4) involved in fumarate formation from succinate was added to the model (Table 1). The protein coding sequence Csac_1640 was predicted to code this enzyme as a result of genome-scale re-annotation [28]. Some of the reactions are essential in the central carbon metabolism to maintain its continuity and is found missing. Genes coding for the enzymes involved in succinate formation from succinyl-CoA (EC: 6.2.1.5) and malate from oxaloacetate (EC: 1.1.1.37) were not found. As a result, these spontaneous reactions were needed to complete the pathway and were included to the model without any gene association, based on the metabolic network of phylogenetic neighbour *Paenibacillus mucilaginosus 3016* (Table 1). Finally, the refined model of central carbon metabolism consists of 31 reactions (Figure 1).

A simplified stoichiometric model of central carbon metabolism pathway of *C. saccharolyticus* was constructed (Figure 2) based on the biochemical information. A cytosolic compartment was applied to ideally depict the influx and efflux rate of substrate, co-factors and product [36]. 31 reactions (excluding influx reactions indicated as "*" and efflux reactions indicated as "#") were assumed to be directly or indirectly involved in H_2 production which was taken further for EMs analysis. In that, 12 and 19 reactions are identified as irreversible (=>) and reversible (<=>) respectively. The complete stoichiometry information of the newly modeled central carbon metabolism pathway is given in Table 2. Based on the biochemical reactions (Table 2) an elementary mode study was carried out for the identification of the best route using METATOOL.

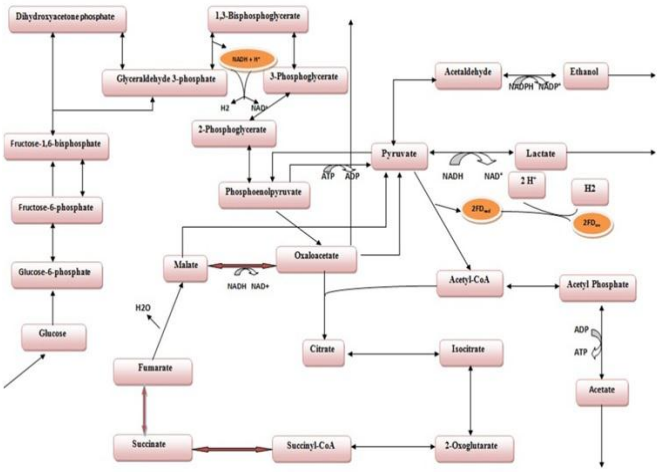

Figure 1. Reconstructed central carbon metabolism of Caldicellulosiruptor saccharolyticus. Reactions newly included in central carbon metabolism after reconstruction are shown in solid red lines.

Table 1. List of reactions newly included in central carbon metabolism after reconstruction.

S. No.	Metabolism	EC Number	Enzyme	Reaction*
1.	Central carbon metabolism	EC:1.3.5.1/ 1.3.5.4	Fumarate reductase/succinate dehydrogenase	Succinate <==> Fumarate
2.	Central carbon metabolism	EC: 6.2.1.5	Succinate---CoA ligase	Succinyl-CoA <==> ATP + Succinate
3.	Central carbon metabolism	EC:1.1.1.37	Malate dehydrogenase	NADH + Oxaloacetate <==> Malate

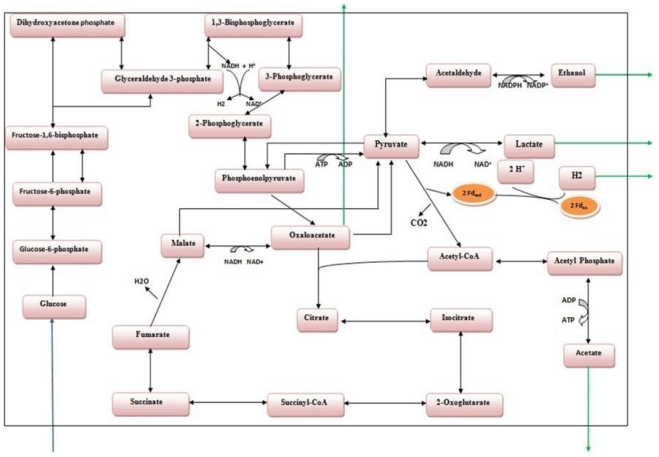

Figure 1. The modeled central carbon metabolism pathway of Caldicellulosiruptor saccharolyticus. The influx and efflux direction of substrate and products are indicated with blue and green color lines respectively.

Table 2. Reactions and enzymes involved in the Central carbon metabolism pathway model of *Caldicellulosiruptor saccharolyticus* taken for elementary modes prediction and gene knockout studies.

Reacn No	Reactions	Gene Name	EC Number	Enzyme
R 1:	==> Glucose	*	*	Influx
R 2:	Acetate ==>	#	#	Efflux
R 3:	Lactate ==>	#	#	Efflux
R 4:	Oxaloacetate ==>	#	#	Efflux
R 5:	Ethanol ==>	#	#	Efflux
R 6:	H_2 ==>	#	#	Efflux
R 7:	ATP + glucose ==> ADP + glucose 6-phosphate	*Glk*	2.7.1.2	Glucokinase
R 8:	D-glucose 6-phosphate <==> D-fructose 6-phosphate	*pgiA*	5.3.1.9	Phosphoglucose isomerase
R 9:	ATP + D-Fructose 6-phosphate ==> ADP + D-Fructose 1,6-	*pfkA*	2.7.1.11	6-phosphofructokinase

Reacn No	Reactions	Gene Name	EC Number	Enzyme
	bisphosphate			
R 10:	phosphate + D-Fructose 1,6-bisphosphate <==> D-Fructose 6-phosphate	*Pfp*	2.7.1.90	Pyrophosphate--fructose 6-phosphate 1-phosphotransferase
R 11:	D-fructose 1,6-bisphosphate <==> Glycerone phosphate + D-glyceraldehyde 3-phosphate	*fbaA*	4.1.2.13	Fructose-bisphosphate aldolase
R 12:	D-glyceraldehyde 3-phosphate <==> Glycerone phosphate	*tpiA*	5.3.1.1	Triose phosphate isomerase
R 13:	D-glyceraldehyde 3-phosphate + phosphate + NAD+ <==> 3-phospho-D-glyceroyl phosphate + NADH + H+	*gapA*	1.2.1.12	Glyceraldehyde-3-phosphate dehydrogenase
R 14:	NADH + H+ ==> NAD + H2	*Hyd*		NADH-dependent Fe-only hydrogenase
R 15:	ADP + 3-phospho-D-glyceroyl phosphate <==> ATP + 3-phospho-D-glycerate	*Pgk*	2.7.2.3	Phosphoglycerate kinase
R 16:	3-phospho-D-glycerate <==> 2-phospho-D-glycerate	*gpmI*	5.4.2.12	Phosphoglycerate mutase
R 17:	2-phospho-D-glycerate <==> Phosphoenolpyruvate + H2O	*Eno*	4.2.1.11	Phosphopyruvate hydratase (enolase)
R 18:	ADP + Phosphoenolpyruvate+ H+ ==> ATP + Pyruvate	*Pyk*	2.7.1.40	Pyruvate kinase
R 19:	Pyruvate + CoA + 2 oxidized ferredoxin <==> Acetyl-CoA + CO2 + 2 reduced ferredoxin + 2 H+	*porG*	1.2.7.1	pyruvate:ferredoxin oxidoreductase
R 20:	ATP + Pyruvate <==> Phosphoenolpyruvate	*Ppdk*	2.7.9.1	Pyruvate phosphate dikinase
R 21:	Pyruvate <==> CO2 + Acetaldehyde		4.1.1.1	Pyruvate decarboxylase
R 22:	H + Acetaldehyde + NADPH <==> Ethanol + NADP	*Adh*	1.1.1.1	Alcohol dehydrogenase
R 23:	Acetyl-CoA + phosphate ==> CoA + Acetyl phosphate	*Pta*	2.3.1.8	Phosphotransacetylase
R 24:	NADH + H+ + Pyruvate <==> NAD + Lactate	*Ldh*	1.1.1.27	Lactate dehydrogenase
R 25:	ADP + H+ + Acetyl phosphate ==> ATP + Acetate	*ackA*	2.7.2.1	Acetate kinase
R 26:	2 H + Fdred ==> Fdox + H_2	*ech*		Ferredoxin-dependent [NiFe] hydrogenase
R 27:	Phosphoenolpyruvate + CO2 <==> Oxaloacetate	*pckG*	4.1.1.32	Phosphoenolpyruvate carboxykinase
R 28:	H + Oxaloacetate ==> Pyruvate + CO2	*oadA*	4.1.1.3	Oxaloacetate decarboxylase
R 29:	NAD + Malate ==> H + Pyruvate + CO2 + NADPH	*Mdh*	1.1.1.38	Malate dehydrogenase
R 30:	Citrate <==> Isocitrate	*acnB*	4.2.1.3	Aconitase
R 31:	Acetyl-CoA + Oxaloacetate ==> Citrate	*CS*	2.3.3.1	Citrate synthase
R 32:	Isocitrate ==> CO2 + NADPH + 2-oxoglutarate	*Idh*	1.1.1.41	Isocitrate dehydrogenase
R 33:	2 Fdox + COA + 2-oxoglutarate ==> 2 H + 2 Fdred + CO2 + Succinyl-CoA	*korD*	1.2.7.3	2-oxoglutarate:ferredoxin oxidoreductase
R 34:	Succinyl-CoA <==> ATP + Succinate		6.2.1.5	Succinate---CoA ligase
R 35:	Succinate <==> Fumarate	*Sdh/ Frd*	1.3.5.1/ 1.3.5.4	Succinate dehydrogenase/ Fumarate reductase
R 36:	H2O + Fumarate <==> Malate	*fumB*	4.2.1.2	Fumarase
R 37:	NADH + Oxaloacetate <==> Malate		1.1.1.37	Malate dehydrogenase

Identification of elementary modes

Elementary Modes are the minimal sets of reactions catalyzed by enzymes that allow the network to perform at steady-state [37,38]. They represent the route of utilizing external substrate and forming external products and thus they are defined in the context of whole-cell metabolism. The modeled central carbon metabolic pathway was further taken for EMs analysis study. In this study, glucose was used as the substrate or carbon source which dissimilates to form a combination of lactate, acetate and H2. These are the major end products formed during fermentation and, pyruvate serves as the major intermediate

with carbon either being routed to lactate or Acetyl-CoA and electrons being routed to lactate or H2.

The resulting network comprises of totally 37 reactions (including influx and efflux reactions) with 40 metabolites (Table 2). A total of 96 EMs were obtained from elementary mode analysis, out of which 67 modes were involved in H2 production. Five EMs: EM 18, EM 25, EM 29, EM 31 and EM 35 were predicted to have minimal reaction sets for H2 production whereas EM 81 was predicted to attain highest flux rate towards H2, from consumption of glucose as substrate (Figure 3 A,B). By examining the above mentioned best EMs the key reactions were identified and used to carry out flux optimization. The stoichiometry matrix of metabolites involved in the modeled central carbon metabolism pathway was generated using CellNetAnalyzer software (Figure 4). The rows of the matrix relate to the metabolites (names displayed on the left boundary of the window) and the columns correspond to the reactions (names shown on the lower boundary of the window).

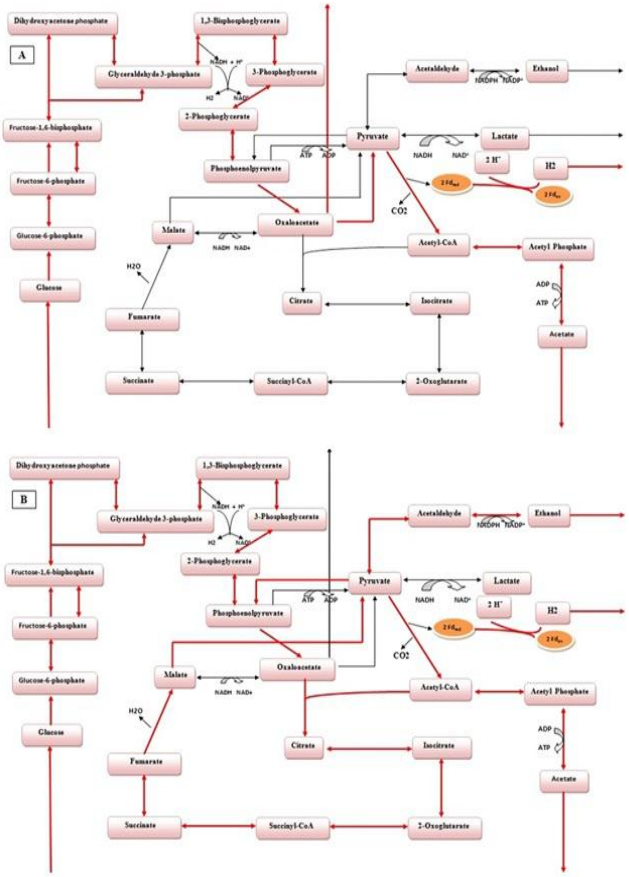

Figure 3. Important EMs of modeled Central carbon metabolism of Caldicellulosiruptor saccharolyticus (A) Figure depicting EM 18 that consists of minimal reaction sets required for hydrogen production. (B) EM 81 of central carbon metabolism predicted to attain maximum hydrogen flux rate.

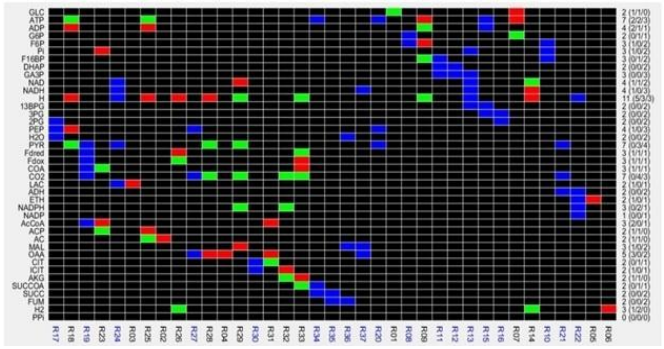

Figure 4. Stoichiometric matrix of central carbon metabolism model. Stoichiometric matrix of central carbon metabolism was constructed with 37 reactions and 40 metabolites. (i) A black matrix signifies metabolites that are neither consumed nor produced in the reaction (ii) A green matrix element corresponds to a metabolite which is produced in an irreversible reaction (iii) A red matrix element indicates a metabolite which is consumed in an irreversible reaction (iv) A blue matrix element corresponds to a metabolite which participates in a reversible reaction (v) The names of reversible reactions are displayed in blue.

Gene participation in elementary modes

Through EMs analysis the involvement of genes in the H_2 production through central carbon metabolism pathway was studied. The gene sets *glk*, *pgiA*, *pfkA*, *fbaA*, *hyd*, *tpiA*, *gapA*, *pgk*, *gpmI*, *porG*, *ech* and *eno* catalyzing reactions with glucose as carbon source, participated in all the 67 EMs involved in H_2 production in central carbon metabolism pathway (Figure 5). The above mentioned gene sets are involved in all the modes and are predicted to be important genes for maintaining the carbon flux rate throughout the pathway and hence cannot be blocked. The genes *oadA* and *pckG* are involved in 61 Ems whereas *adh* and *pfp* is involved in 53 and 44 EMs respectively. The gene sets *acnB*, *idh*, *korD*, *fumB*, *sdh/frd* and *CS* were involved in 42 EMs. The gene sets *mdh*, *pta*, *ackA*, *ppdK* participated in less than 40 EMs. The genes *pyk* and *ldh* were found to be involved in the least number of EMs, that is, 19 and 8 EMs respectively (Figure 5). These findings suggest that blocking the gene sets or reactions that are involved in the least number of EMs does not affect carbon flow during central carbon metabolism pathway.

Hydrogen formation occurs in 67 EMs, 8 of which are related to the production of lactate and 26 EMs are involved in acetate formation which are the other major fermentative products formed during central carbon metabolism. Thus, if the genes related to H_2 will be removed, the formation of lactate and acetate will also be affected. This is the first elementary flux mode analysis work reported in *C. saccharolyticus* for the purpose of enhancing H_2 production. EMs (minimal gene sets) analysis helped in identifying the key reactions that play role in H_2 production. As a result of flux optimization, the theoretical molar yield of H_2 has been calculated under different EM stages. From the EM analysis, the knockout of genes which

are involved in more number of EMs affects the H_2 production indicating the importance of these genes in the carbon flow towards Acetyl-CoA. The elementary mode study of central carbon metabolism helped in identifying the involvement of each gene in H_2 production. This study also helped us in identifying the possible gene knockouts which will further help in optimizing the glucose intake towards hydrogen formation.

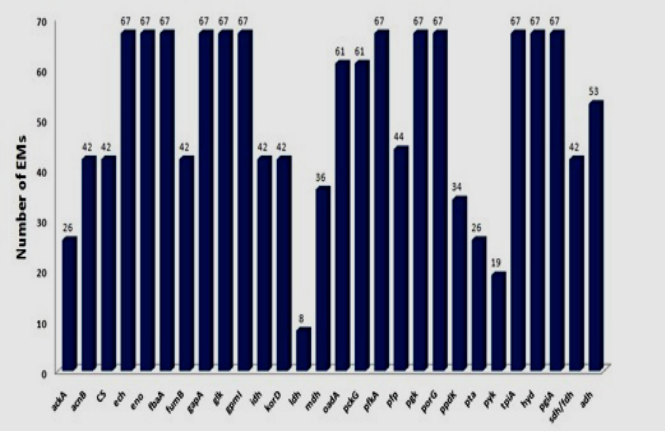

Figure 5. Histogram showing the number of elementary modes each gene is involved.

In silico gene knockout analysis

In *C. saccharolyticus* hydrogenase genes utilizes reduced electron carriers NADH and Fd_{red} for proton reduction, thus forming H_2. Since the H_2 evolution from NADH is thermodynamically unfavorable except in extremely low H_2 partial pressures, compared to that from ferredoxin [39], we focussed on enhancing the flux rate towards H_2 evolved from the latter, by gene knockout analysis. EMs analysis helped in studying gene knockout strategies. Measurement of flux using experimental analysis consumes a lot of time and effort [40]. Thus, using the reconstructed model, *in silico* flux analysis and gene knockout studies has been carried out. The modeled central carbon metabolism has been used to identify the gene knockout candidates for enhancing the H_2 production. The single gene deletion, as well as double gene deletions, have been studied. The *in silico* gene deletion for the metabolism is carried out by setting the constraints to zero for a gene to be knocked out and thus removing the enzymatic reaction from the stoichiometric matrix of the metabolism. A total of 67 EMs were observed and their fluxes were measured. Among them, EM 81 gene sets or reaction flow has maintained the maximum possible yield of H_2 and retained a reasonable yield of biomass while the other EMs with minimal product fluxes were eliminated.

The modeled central carbon metabolism pathway (Figure 2) has been used to achieve the enhanced theoretical molar yield of H_2. An *in silico* gene knock-out of lactate dehydrogenase (*ldh*) gene was performed during flux optimization of EM 81, which yielded an increase in the flux of hydrogen from 34.2557 to 45.4689 *mmol/gDW/hr* (Figure 6). A double gene knockout of lactate dehydrogenase (*ldh*) and pyruvate kinase (*pyk*) yielded an increase in H_2 flux rate to 46.2758 *mmol/gDW/hr* (Figure 7). The enzymes lactate dehydrogenase and pyruvate kinase have been knocked out since it is not involved in EM 81 which was predicted to attain higher flux rate towards H_2 and were found to participate in the least number of EMs as well. The calculated fluxes of major metabolites H_2, acetate and lactate from EM 81 without gene knockout, with single and double gene knockout is depicted in Figure 8.

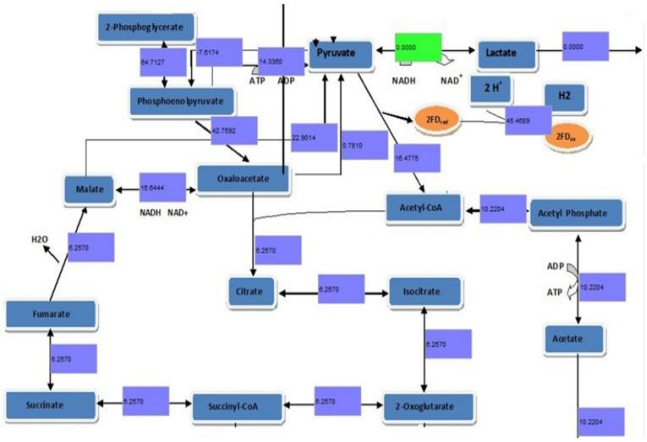

Figure 6. Single gene knockout on EM 81 of modeled central carbon metabolism of Caldicellulosiruptor saccharolyticus. The measured flux rate was shown as blue color box and the gene knocked out was highlighted as green color box.

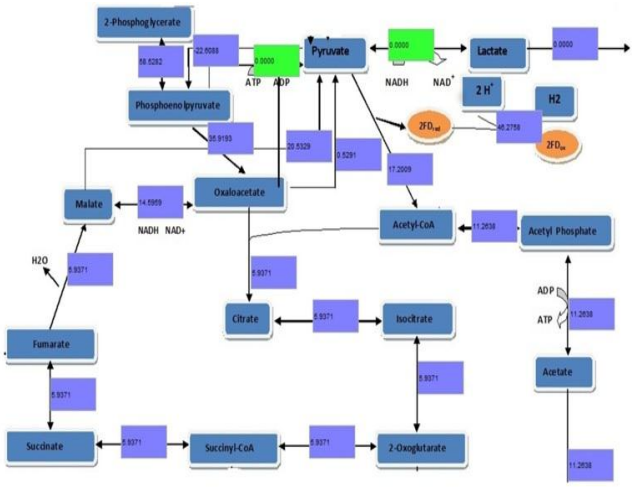

Figure 7: Double gene knockout on EM 81 of modeled central carbon metabolism pathway of Caldicellulosiruptor saccharolytics. The measured flux rate was shown as blue color box and the genes knocked out was highlighted as green color box.

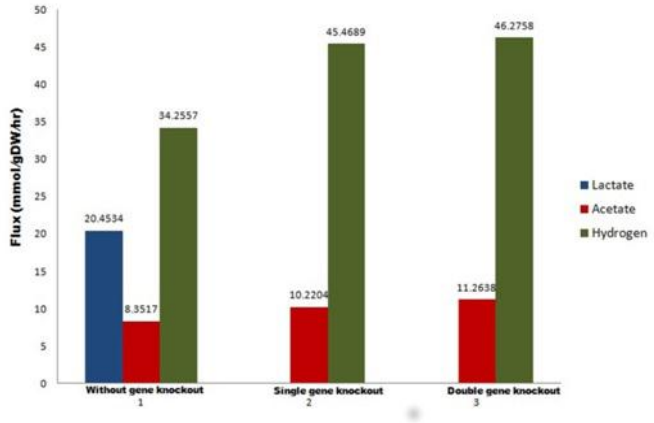

Figure 8. The calculated fluxes of hydrogen, acetate and lactate during central carbon metabolism of Caldicellulosiruptor saccharolyticus (1) Graph represents the flux rates of metabolites without gene knockout. (2) The maximum fluxes obtained from EM 81 by knockout of ldh gene (single gene knockout). (3) Flux rates obtained from EM 81 with double gene knockout (ldh and pyk genes).

IV. DISCUSSION

The computational effort for the analysis of genome-scale models has increased with their increasing size and complexity. Due to computational intractability, some stoichiometric methods that demand the enumeration of elementary modes [14], cannot be applied to networks comprising of a few thousand reactions [41]. Therefore, elementary mode flux analysis was carried out on a small network (central carbon metabolism) of *C. saccharolyticus* which helped us to identify the potential genes to be knocked out for enhancing hydrogen production. Members of the genus *Caldicellulosiuptor* are hyperthermophilic that converts biomass to products of interest and they are capable of using biomass without conventional pretreatment [39]. During growth on glucose, *C. saccharolyticus* utilizes the Embden-Meyerhof glycolytic pathway and produces a combination of lactate, acetate and hydrogen as major fermentative products. Our strategy for improving hydrogen production involved the modification of energy metabolism, to direct the flow of major metabolites like hydrogen through elementary flux mode analysis. The biochemical reactions present in central carbon metabolism pathway is clearly illustrated in Figure 1 and the functions of corresponding genes are summarized in Table 2.

An increase in production of acetate and hydrogen by 21-34% relative to the wild-type and ΔpyrFA parent strains has been reported with *ldh* gene deleted *Caldicellulosiruptor bescii* strain which is the first targeted gene deletion study for metabolic engineering of a member of the genus *Caldicellulosiruptor* [39] Furthermore, the *ldh* deletion strain reported a longer exponential growth phase relative to the wild type in *C. bescii* [39]. In the present study, single gene knockout (*ldh*) and double gene knockout (*ldh* and *pyk*) obtained from elementary mode analysis were hypothesized to increase the flux rate of hydrogen production in EM 81 (predicted to attain higher flux rate towards hydrogen). An increase in the flux of hydrogen from 34.2557 to 45.4689 *mmol/gDW/hr* has been reported with *ldh* gene knockout. Similarly, a double gene knockout (*ldh* and *pyk*) yielded an increase in hydrogen flux rate to 46.2758 *mmol/gDW/hr* in EM 81. As discussed above, to improve the hydrogen yield by *C. saccharolyticus*, knockout of *ldh* and *pyk* genes in central carbon metabolism pathway would be of prior consideration.

V. CONCLUSION AND FUTURE SCOPE

Here, we report the first *in silico* elementary mode flux balance analysis work in *C. saccharolyticus*. Under controlled intake of glucose, metabolic perturbations resulting from single and double gene knockouts led to strongly increase the flux rate of H_2 formation. These results will help us to get an in-depth understanding of central carbon metabolism in order to develop this organism as a potential source for H_2 production. The *in silico* gene knockout and flux analysis results presented here will further help in the improvement of strain through metabolic engineering strategies for obtaining an enhanced H_2 producing phenotype.

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