

MODELING THE OPTIMAL CENTRAL CARBON METABOLIC PATHWAYS UNDER FEEDBACK INHIBITION USING FLUX BALANCE ANALYSIS

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Metabolism is a complex process for energy production for cellular activity. It consists of a cascade of reactions that form a highly branched network in which the product of one reaction is the reactant of the next reaction. Metabolic pathways efficiently produce maximal amount of biomass while maintaining a steady-state behavior. The steady-state activity of such biochemical pathways necessarily incorporates feedback inhibition of the enzymes. This observation motivates us to incorporate feedback inhibition for modeling the optimal activity of metabolic pathways using flux balance analysis (FBA). We demonstrate the effectiveness of the methodology on a synthetic pathway with and without feedback inhibition. Similarly, for the first time, the Central Carbon Metabolic (CCM) pathways of *Saccharomyces cerevisiae* and *Homo sapiens* have been modeled and compared based on the above understanding. The optimal pathway, which maximizes the amount of the target product(s), is selected from all those obtained by the proposed method. For this, we have observed the concentration of the product inhibited enzymes of CCM pathway and its influence on its corresponding metabolite/substrate. We have also studied the concentration of the enzymes which are responsible for the synthesis of target products. We further hypothesize that an optimal pathway would opt for higher flux rate reactions. In light of these observations, we can say that an optimal pathway should have lower enzyme concentration and higher flux rates. Finally, we demonstrate the superiority of the proposed method by comparing it with the extreme pathway analysis.

Keywords: Feedback inhibition; central carbon metabolic pathways; flux balance analysis; extreme pathway analysis; metabolic engineering.

1. Introduction

Biochemical conversions in metabolic pathways are mostly catalyzed by enzymes. Enzymes allow these reactions to proceed quickly and efficiently to yield an adequate concentration of a target product necessary in a cell for its normal functioning. However, if the concentration of the target product exceeds that required by a cell,

then it may lead to malfunction. Thus there should be a regulatory mechanism to prevent this overproduction. In other words, the biochemical conversions need to be shut off to avoid such overproduction. In a kind of regulation, the product apply a regulation, the product itself binds with the enzyme for its inhibition. This kind of reaction occurs at a site, called the allosteric site, on the enzyme that is different from its active site. Then the enzyme undergoes a conformational change and can no longer act as a catalyst. In this way, the synthesis of the target product is controlled. In other words, cells have the ability to shut down a pathway when a target metabolite is not needed. This shut down or control process is carried out through several ways, including concerted feedback, sequential feedback and cumulative/cooperative feedback.¹ However, the most common way of control is inhibition of the initial reaction by the final product of the pathway (end-product inhibition or overall feedback inhibition).

There exist several types of metabolic feedback regulation, including control of metabolite concentration by enzymes,² control of enzyme mRNA transcription,³ reversible enzyme phosphorylation,⁴ non-competitive allosteric regulation⁵ and competition for enzyme active sites.⁶ The control by molecular feedback inhibition in biochemical pathways was initially discovered in unbranched biosynthetic pathways.^{7,8} Several approaches for *in silico* analyses of feedback inhibition include the study of a control pattern in which a sequence of feedback inhibitions occur and each intermediate inhibits the reaction that immediately precedes it⁹; overall feedback inhibition, which magnifies the flux regulation and decreases the sensitivity of the concentrations for the demand of end product¹⁰; exhibition of limit-cycle oscillations in a coupled network with product-feedback inhibition¹¹; and demonstration of simple feedback inhibition for optimal and efficient biomass production in a metabolic pathway.¹² The study¹⁰ used a method for comparing the function of alternative molecular designs and its analytical results show that the unbranched pathway can achieve the same steady-state flux, concentrations and logarithmic gains with respect to changes in substrate, with or without overall feedback inhibition. Although the study does not show the effect of overall feedback inhibition on the robustness, stability, and transient time of the pathway, it shows that stable systems with overall feedback inhibition respond faster to fluctuations in the metabolite concentrations.

Computer simulations have been used to map the utilization of enzymatic capacity, flux control and response time in a metabolic network regulated by non-allosteric feedback inhibition. It has been found that these network properties behave in an antagonistic manner.¹³ The investigations shows that the response time is based on the time required for the system to reach 95% of its next steady state following a step change in an intermediate metabolite concentration. Here, small response time refers to quick adaptation, which is advantageous for a biological network. This study has revealed that the dynamics of a regulated system would differ and significantly affect the response time. It has also been found that apparent trade-offs occur between definable objectives, if one examines the abovementioned

properties as a function of the network's operating and structural characteristics. Thus it has been suggested that the consideration of trade-offs helps in the interpretation of functional role of metabolic networks.

The method given in Ref. 2 uses the connectivity theorem for enzymes that control metabolite concentrations, unlike the present approach that modifies FBA for feedback inhibition. Goyal *et al.*¹² have studied the feedback inhibition on linear and bidirectional pathways and on a metabolic cycle and found that simple product-feedback inhibition helps in the optimal biomass production, though it also leads to the accumulation of toxic metabolite pools. The method in Ref. 14 is concerned with flux distribution based on maximizing the rate of total entropy production during adaptive evolution. Here, the authors have described a metabolic network through a set of elementary modes and shown that the usage probability of individual elementary modes is distributed according to the Boltzmann distribution law in the most likely metabolic state. To support this fact, they have shown that the evolving mutant of *Thermoanaerobacterium saccharolyticum* adjusts the metabolic flux distribution during adaptive evolution in a direction of increasing rate of entropy product. However, this study does not consider feedback inhibition.

Flux Balance Analysis (FBA) is found to be useful for large-scale analysis of metabolic, signaling and gene regulatory pathways.^{15–17} It utilizes the stoichiometric matrix and a biologically relevant objective function to identify optimal reaction flux distributions. The objective function is to be maximized (biomass) or minimized (toxin expression by a pathogen) according to the requirement of the study. This needs minimal amount of biological knowledge and reaction parameters required to make quantitative inferences about network behavior. In the present study, we develop a methodology under FBA to identify optimal metabolic pathways in terms of the level of concentration of the enzymes catalyzing various reactions in the entire metabolic network for both with feedback inhibition and without feedback inhibition conditions. Here the optimal pathways mean the pathways that lead to the production of the maximum amount of target product. The methodology is a modification of an existing one,¹⁸ where a method has been developed to determine optimal metabolic pathways in terms of the level of concentration of the enzymes catalyzing various metabolic reactions. Unlike this method,¹⁸ the present method considers feedback inhibition that exists in real-life pathways. We also distinguished between the functional nature of the enzymes present in the pathway. There are two kinds of enzymes present: (i) enzymes catalyzing the metabolic reactions, and (ii) enzymes responsible for both catalyzing the reactions and regulating the metabolism in the system. Here we provide a novel formulation for finding the concentration of these regulating enzymes present in a pathway. We made the following hypotheses: (i) The concentration of regulatory enzymes will be decreased in the feedback inhibited pathway compared to that in the pathway without feedback inhibition. This is due to the fact that the target product/intermediate substrate inhibits its own production through binding with

its corresponding/regulatory enzymes. (ii) The optimal concentrations of the enzymes that catalyze the reactions to synthesize the target product will be lower in the feedback inhibited pathway compared to that in the pathway without feedback inhibition. (iii) The metabolic reactions with increased flux values will be opted by an optimal pathway to increase the production of the target metabolite. The effectiveness of the methodology along with comparison with Extreme Pathway Analysis (EPA)^{19,20} had been demonstrated on CCM pathways of *Saccharomyces cerevisiae* and *Homo sapiens*. A brief description of CCM pathways of *S. cerevisiae* and *H. sapiens* is provided in Sec. 2.

The optimal pathways obtained for CCM pathways with and without feedback inhibition for *S. cerevisiae* and *H. sapiens* had been found to be different. We performed the following studies on both these organisms. We studied and compared the concentrations of the regulating enzymes in both of the aforesaid conditions. We also considered the concentrations of the enzymes that catalyze the reactions for target metabolites. Under the condition of feedback inhibition, we hypothesized that the concentrations of these regulating enzymes would be lower as the system does not need higher amount of the product metabolite after a certain time. In other words, the amount of material flowing through the pathway is intimately coupled to the metabolic needs of the cell.^{10,21} The results justified this hypothesis as the concentrations of most of these enzymes have been found to be lower. We also observed that an optimal pathway will opt for higher flux rates for quicker synthesis of the products. We compared the results obtained for optimal CCM pathways of *S. cerevisiae* and *H. sapiens* with and without feedback inhibition conditions. The results obtained by the proposed method for optimal CCM pathways of *H. sapiens* in both aforementioned conditions were further compared with EPA. The major drawback with the EPA algorithm is that it does not consider enzyme concentration. Enzyme concentration should be considered in the study as it influences the production of the target product. This is an essential step to make the study closer to real-life situations.

2. A Brief Description of CCM Pathways of *S. cerevisiae* and *H. sapiens*

Yeast can metabolize sugar in two ways *viz.*, aerobically or anaerobically. Among these, there is a category that metabolizes glucose during aerobic cultivation and produces ethanol, known as Crabtree positive yeast *e.g.*, *S. cerevisiae* catabolizes glucose mainly by a fermentative process, and this effect is called as Crabtree effect.^{22,23} In *S. cerevisiae* batch cultures, when the levels of glucose decline, cells become gradually derepressed, resulting in the induction of respiratory enzyme synthesis. This in turn results in the oxidative consumption of ethanol, when cells enter a second phase of growth known as the diauxic shift. The *S. cerevisiae* CCM pathway has a junction where fermentative and respiratory sugar metabolism diverge, *i.e.*, the pyruvate branch-point. Most of the glucose-6-phosphate is directed

through glycolysis that further forms pyruvate.²² At the pyruvate branching point, the carbon is distributed between the respiratory and fermentative pathways. Pyruvate can follow three different fates, depending on the yeast species and the environmental conditions,²⁴ viz., (1) Under respiratory conditions, pyruvate is transported into the mitochondria where it is converted to acetyl CoA by the mitochondrial pyruvate dehydrogenase enzyme complex.²⁵ (2) Oxaloacetate (OAA) can be produced from cytosolic pyruvate. (3) During fermentative conditions, pyruvate is decarboxylated to acetaldehyde by the enzyme pyruvate decarboxylase. Acetaldehyde can either be reduced to ethanol by the enzyme alcohol dehydrogenase²⁶ or oxidized to acetate by the enzyme aldehyde dehydrogenase.²⁷ In contrast to this, the fate of pyruvate is different in aerobic organisms. Pyruvate produced in glycolysis is oxidized to CO₂ via Tri Carboxylic Acid (TCA) cycle, and the NADH produced in glycolysis and TCA cycle is reoxidized via the respiratory chain, with production of additional ATPs.

Central Carbon Metabolism (CCM) is one of the key metabolic modules governing energy sources and creating primary building blocks of other metabolisms and is a cornerstone of metabolic processes. CCM is a fundamental process of life, which uses a complex series of enzymatic steps to convert sugars into metabolic precursors.²⁸ Organisms metabolize some nutrients in cyclic form of a pathway, which can be described as a wrapped linear pathway where the end product is essential for the first step of the pathway *e.g.*, TCA cycle in carbon metabolism. In such cases, the import of nutrients is slowed or stopped if there is not enough end product available. Therefore, an adequate amount of the end product must always be maintained in order to achieve optimal growth. We have described the cyclic nature of TCA as it is a part of the CCM pathway. The interchange of carbon atoms between glycolysis pathway and pentose phosphate pathway (PPP) takes place at the level of fructose-6-phosphate and/or glyceraldehyde-3-phosphate. PPP is also interconnected with the pathways, those are related mainly to nucleotide and lipid metabolism.²⁹ The anabolic or catabolic functions of PPP may depend on the entrance or exit of metabolites. Further metabolism of glucose connected with PPP is carried out by the classical glycolysis/gluconeogenic pathways.

3. Methodology

Here we describe the proposed methodology for incorporating product feedback inhibition on enzymatic activity under FBA. Then it is used to determine optimal CCM pathways of *S. cerevisiae* and *H. sapiens*, through which the amount of target product(s) is maximum. We have considered the following cases — with and without feedback inhibition of the metabolites on enzymatic activity. The methodology is a modified form of an earlier one¹⁸ in which the authors have not considered the case of such feedback inhibition. The proposed methodology is depicted in Fig. 1.

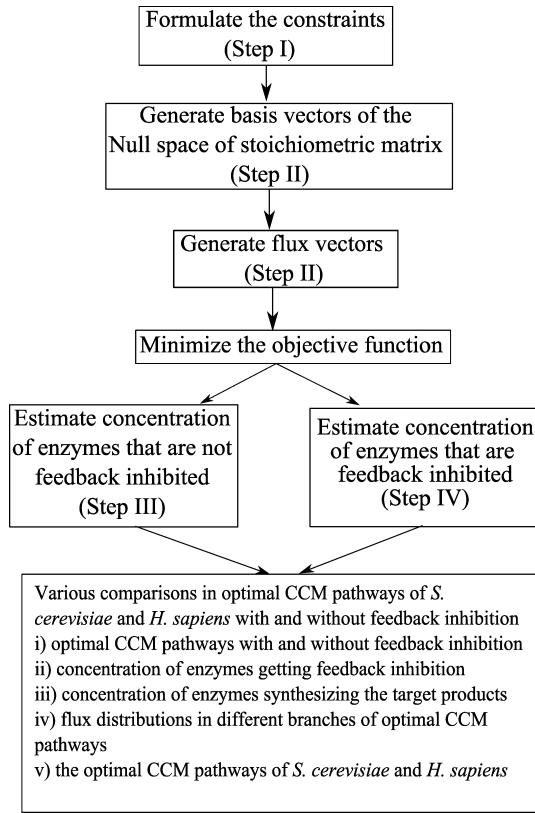


Fig. 1. Flow of the work done.

3.1. Step I: Formulating the constraints

We start with writing the mass balance constraint under steady state condition using FBA framework *i.e.*, $\mathbf{S} \cdot \mathbf{v} = \mathbf{0}$. Here $\mathbf{S} = [S'_{ij}]_{m \times n}$ is the stoichiometric matrix of order $m \times n$, where m is the number of metabolites and n is the number of reactions in the network. The vector \mathbf{v} represents a possible flux vector in the metabolic network. Since the flux of a reaction depends on concentration of the enzyme catalyzing it, this equation has been modified in Ref. 18 as $\mathbf{S}(\mathbf{C}\mathbf{v}) = \mathbf{0}$. The term, \mathbf{C} is an $n \times n$ diagonal matrix whose diagonal elements are the components of the vector \mathbf{c} . The term c_j , in $[0,1]$, is j th component of \mathbf{c} , and represents the concentration of the enzyme catalyzing j th reaction with flux v_j . This modification places emphasis on the restrictions found in real-life systems. For example, it is possible in biological systems that a gene that produce the enzyme may not be expressed at the desired level.

3.2. Step II: Generating flux vectors

As described in Ref. 18, we generate those flux vectors that satisfy the standard state condition *i.e.*, $\mathbf{S} \cdot \mathbf{v} = \mathbf{0}$. In other words, we generate those flux vectors that are

possible when the concentrations of the enzymes catalyzing the corresponding reactions are at the desired/highest level. For this purpose, we first generate basis vectors \mathbf{v}_b that form the null space of the stoichiometric matrix \mathbf{S} . Let the number of such basis vectors be l . Then we generate a vector, $\mathbf{v} = \sum_{p=1}^l \alpha_p v_{bp}$, $\epsilon_p \in [0, 1]$ being an arbitrary random number, until certain inequality constraint on \mathbf{v} is satisfied for all its components. The inequality constraint for an internal flux is $v_j \geq 0, \forall j$.³⁰ The constraints on the exchange fluxes depend on their directions and can be shown as $\alpha_j \leq v_j \leq \beta_j$, where α_j and β_j are either zero, or negative and positive infinity, respectively based on the direction of the exchange flux. The values of α_j and β_j are based on the direction of the exchange flux. The sign of an exchange flux is considered to be positive if the metabolite is exiting or being produced by the system, and negative if the metabolite is entering or being consumed by the system. Detailed explanation can be found in Ref. 30. Here, a large number of such flux vectors \mathbf{v} are generated to form the data set. Next we formulate the objective function for with and without feedback inhibition conditions.

3.3. Step III: Estimation of concentration of enzymes that are not feedback inhibited

Now the objective function can be written as in Ref. 18,

$$y = \frac{1}{z} + \mathbf{\Lambda}^T \cdot (\mathbf{S} \cdot (\mathbf{C} \cdot \mathbf{v})) \quad (1)$$

This y needs to be minimized with respect to c_j for all j . The term $z = \sum_{j=1}^s c_j v_j$ represents an objective function which gives the total amount of the target metabolites. As in the case of FBA, we take the algebraic sum over the reactions R_1, R_2, \dots, R_s that directly yield the target molecule, and is given by a above-mentioned linear equation. It needs to be maximized or minimized according to the problem. In the present case, we maximize the amount of the target molecules *i.e.*, minimize y .

The regularizing parameter $\mathbf{\Lambda} = [\lambda_1, \lambda_2, \lambda_3, \dots, \lambda_m]^T$ is such that $0 < \lambda_j \leq 1, \forall j$. Here we assume that $\lambda_1 = \lambda_2 = \dots = \lambda_m = \lambda$ (say), for simplicity. Minimization of y is carried out by adopting the gradient descent technique given in Ref. 31. Initially, a set of random values in $[0, 1]$ corresponding to c_j -values are generated. The c_j -values are then modified iteratively using the gradient descent technique, where the amount of modification for c_j in each iteration is defined by the equation given below, so that c_j value is modified to $c_j(t) + \Delta c_j$. The term η is a small positive quantity indicating the rate of modification.

$$\Delta c_j = -\eta \frac{\delta y}{\delta c_j} \quad (2)$$

The modified value of c_j is given by the following equation

$$c_j(t+1) = c_j(t) + \Delta c_j, \quad \forall j, t = 0, 1, 2, \dots \quad (3)$$

Here $c_j(t + 1)$ is the value of c_j at iteration $(t + 1)$ which is computed based on the c_j -value at the iteration t . Regularization parameter λ is chosen empirically *i.e.*, by varying the value of λ from 0.1 to 1.0 in steps of 0.1. Using the aforesaid method, for each value of λ , finally we get c_j -values for which y attains a minimum value. We choose c_j -values for which y is minimum over all its minima.

$$\Delta c_j = \frac{1}{z^2} \frac{\delta z}{\delta c_j} - \frac{\delta}{\delta c_j} (\mathbf{\Lambda}^T \cdot (\mathbf{S} \cdot (\mathbf{C} \cdot \mathbf{v}))) \tag{4}$$

From the above equation, we get,

$$\frac{\delta y}{\delta c_j} = -\frac{1}{z^2} \frac{\delta z}{\delta c_j} + \lambda \sum_{i=1}^s S_{ij} v_j \tag{5}$$

The equation for calculating values of Δc_j is given below,

$$\Delta c_j = \eta \left[\frac{1}{z^2} \frac{\delta z}{\delta c_j} - \lambda \sum_{i=1}^s S_{ij} v_j \right] \tag{6}$$

3.4. Step IV: Estimation of concentration of enzymes that are feedback inhibited

In order to simulate feedback inhibition of metabolites on enzymatic activity, we consider a hypothetical simple linear reaction system as given in Fig. 2. Here A is the starting substrate and D is the target metabolite. There are five reactions/conversions R_1, R_2, R_3, R_4 and R_5 in the system. The reactions R_2, R_3 and R_4 are catalyzed by the enzymes E_1, E_2 and E_3 , respectively. We consider inhibition by the metabolite B on the enzyme E_1 . Under this condition, the modified reaction system is given in Fig. 3. During feedback inhibition, B and E_1 form a complex through the reaction R_7 , and then the complex BE_1 goes out of the system through the reaction R_8 . Thus we have to incorporate more fluxes in the flux vectors. Since a certain amount of E_1 becomes ineffective due to its binding with B, the corresponding enzyme concentration will be reduced. The reduction factor depends on the rate of formation of B and thus is assumed to be proportional to rate of formation of B *i.e.*, the corresponding reaction flux. In other words, if the reaction flux through which B is produced is v , then E_1 is reduced by av , $a \in [0, 1]$ being constant of proportionality. If we assume the initial concentration of E_1 is at the desired/highest level *i.e.*, unity, the effective concentration of E_1 becomes $(1 - av)$. This $(1 - av)$ fraction of E_1 is now catalyzing the reaction for formation of B.

Thus the second term on the right-hand side of Eq. (6) becomes $\lambda \sum_{i=1}^s S_{ij}(1 - av_j)v_j$, and is written as

$$\Delta c_j = \eta \left[\frac{1}{z^2} \frac{\delta z}{\delta c_j} - \lambda \sum_{i=1}^s S_{ij}(1 - av_j)v_j \right] \tag{7}$$

where the enzyme for j th reaction is feedback inhibited. However, Eq. (6) is applicable to the enzymes that are not feedback inhibited. It is to be mentioned here that a is a user-defined constant.

4. Results

Here we describe the results obtained by the present method. For this purpose, we considered a synthetic metabolic pathway (Fig. 2), and the Central Carbon Metabolic (CCM) pathways of *S. cerevisiae* and *H. sapiens* (Figs. 4 and 7). CCM is a fundamental process of metabolism. We included glycolysis pathway, TCA cycle and PPP of both the organisms and downloaded from KEGG pathway database (<http://www.genome.jp/kegg/>) to form CCM pathways for both of the organisms. We kept the test case simpler as we included only carbohydrates and phosphate groups (and CoA) as parts of the CCM pathway. We made the following hypotheses.

- The concentration of regulatory enzymes will be decreased in feedback inhibited pathway compared to that in the pathway without feedback inhibition. This is due to the fact that the target product/intermediate substrate inhibits its own production through binding with its corresponding/regulatory enzymes.
- The optimal concentrations of the enzymes that catalyze the reactions to synthesis the target product will be lower in feedback inhibited pathway compared to that in the pathway without feedback inhibition.
- The metabolic reactions with increased flux values will be opted by an optimal pathway to increase the production of the target metabolite.

We performed various analyses based on the results. Following is the organization of Sec. 4. Section 4.1 describes the results obtained from implementing the present method on a synthetic system. Application of the proposed methodology on CCM pathways of *S. cerevisiae* and *H. sapiens* with and without feedback inhibition has been given in Secs. 4.2 and 4.3, respectively. We compared the optimal CCM pathways of *S. cerevisiae* and *H. sapiens* with and without feedback inhibition conditions in Sec. 4.4. We also compared the results obtained for the optimal CCM pathways *H. sapiens* with EPA as given in Sec. 4.5. Section 4.6 involves the possible biological validation for the results obtained for CCM pathways of *S. cerevisiae* and *H. sapiens*.

4.1. On the synthetic system

We considered a synthetic system which had four metabolites, five reactions (three internal fluxes and two external fluxes) for studying a pathway, without feedback inhibition, as shown in Fig. 2. Our objective was to compare the yield of the target product and optimal enzyme concentration in feedback inhibited and without feedback inhibited pathways.

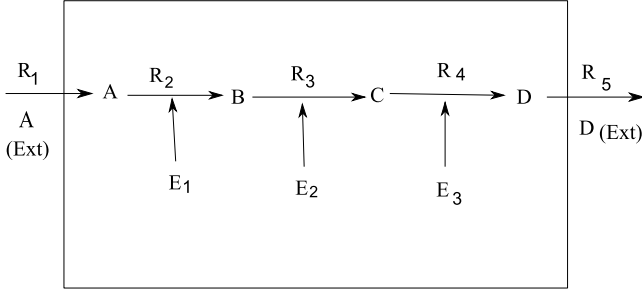


Fig. 2. A synthetic reaction system without feedback inhibition. Here “Ext” refers to external reaction.

4.1.1. Without feedback inhibition

In order to maximize the yield of target product D on substrate A, the objective function z was maximized, where $z = c_4 v_4$. Here, c_4 denotes the concentration of an enzyme E_3 and v_4 stands for the rate of reaction $C \rightarrow D$. Initially, we gave stress on the maximization of the rate of yield not on the constraint. That is, initially λ had been kept small. As we go from $\lambda = 0.1$ to $\lambda = 1.0$, it implies that we are increasing the stress on the constraint, and finally both the rate of yield (z) and the constraint are treated equally. For each value of λ , we minimized y , and considered that set of c_j -values corresponding to the λ -value as the final solution, for which y becomes minimum. The enzyme concentrations for this case had been found to be $c_1 = 0.99008$, $c_2 = 0.78886$ and $c_3 = 0.48103$. Here, c_1 , c_2 and c_3 are the concentrations of the enzymes E_1 , E_2 and E_3 , respectively. Since, it is a linear pathway, all the enzymes concentrations were, as expected, close to 1. The amount of the target product came out as $z = 88.871$.

4.1.2. With feedback inhibition

In the synthetic system, we considered that the enzyme E_1 was inhibited by the product B, as shown in Fig. 3, if amount of the target product D had already attained at the required level in a cell. The product B binds with the enzyme E_1 and inhibits the catalysis of the reaction $A \rightarrow B$. This, in effect, limits the production of the target product D. We considered that the substrate–enzyme complex will go outside the system in feedback inhibited pathway. Thus there were six metabolites and eight reactions (four internal fluxes and four external fluxes). Here the concentrations of the enzymes had been found to be: $c_1 = 0.82703$, $c_2 = 0.95684$ and $c_3 = 0.56106$, and the corresponding yield of the target product (z) was 195.8928. Here, c_1 , c_2 and c_3 are the concentrations of the enzymes E_1 , E_2 and E_3 , respectively. As expected, the concentration of E_1 was lower than that of E_2 for the case of without feedback inhibition. That is, system needs less amount of enzyme if it has enough amount of the target product D, and then it shuts off the pathway. The concentrations of the other enzymes E_2 and E_3 were found to be increased because they remained unused as E_1 was inhibited.

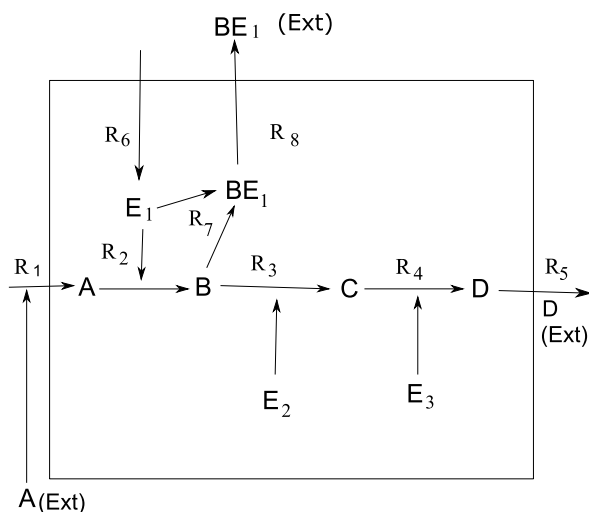


Fig. 3. A synthetic reaction system, in Fig. 2, with feedback inhibition. Here the substrate B binds with the enzyme E_1 , and inhibits its own production hence, the production of the target metabolite D is decreased. Here “Ext” refers to external reaction.

4.2. Application of the proposed methodology on CCM pathway of *S. cerevisiae*: With and without feedback inhibition

The constructed *S. cerevisiae* CCM pathway contained 50 metabolites and 66 reactions (Fig. 4). Our method determined an optimal set of concentrations of the enzymes that was required to get the rate of production of a target metabolite was maximum. In other words, the method is able to determine a set of enzyme concentrations up to which the corresponding enzymes need to be expressed for maximizing the production of the target metabolite grown on a substrate. The considered starting product was, α -D-glucose and target products were glyceraldehyde-3P, ethanol and OAA. We compared the results for the optimal CCM pathways, with and without feedback inhibition.

4.2.1. Without feedback inhibition

The combined yield of ethanol, glyceraldehyde-3P and OAA (target metabolites), in *S. cerevisiae* CCM pathway was maximized. The combined yield of the target products (z) was 44.5269.

4.2.2. With feedback inhibition

The number of metabolites and reactions were 64 and 88, respectively, considering enzyme–substrate complexes for feedback inhibition in CCM pathway of *S. cerevisiae*. The enzymes being inhibited by the above-metabolites and their

Table 1. Inhibited enzymes and their concentrations (*c*-values) in CCM pathways of *S. cerevisiae* and *H. sapiens*, with and without feedback inhibition.

Inhibited enzymes	Inhibiting metabolites	<i>c</i> -values in pathway without feedback inhibition of <i>S. cerevisiae</i>	<i>c</i> -values in feedback inhibited pathway of <i>S. cerevisiae</i>	<i>c</i> -values in pathway without feedback inhibition of <i>H. sapiens</i>	<i>c</i> -values in feedback inhibited pathway of <i>H. sapiens</i>
Hexokinase EC:2.7.1.1	α -D Glucose 6P	—	—	0.90879	0.56593
Phosphofructokinase EC:2.7.1.11	β -D-Fructose-1,6P2	0.50248	0.69438	0.89632	0.87009
Pyruvate kinase EC:2.7.1.40	Pyruvate	0.42682	0.34566	0.99406	0.39084
α -ketoglutarate dehydrogenase EC:1.2.4.2	Succinyl-CoA	1	0	0.45222	0.33465
Citrate synthase EC:2.3.3.1	Citrate	0.42676	0.75344	0.81269	0.6767
Isocitrate dehydrogenase EC:1.1.1.42	2-Oxoglutarate	0.72048	0.37534	0.39334	0.1501
Glucose-6-phosphate dehydrogenase EC:1.1.1.49	D-Glucono-1,5-lactone-6P	0.88076	0.30931	0.74164	0.70614

corresponding concentrations in both the aforesaid conditions are given in Table 1. The combined yield of the target products (*z*) had been found to be 11.8753.

The optimal pathways obtained for *S. cerevisiae* CCM pathway with and without feedback inhibition are shown in Fig. 4. These optimal paths had been found to be different in many aspects. The starting metabolite was α -D glucose in both of the conditions. The given CCM pathway has bifurcation at the metabolite α -D-glucose-6P which can synthesize the metabolite α -D-glucose in feedback inhibited CCM pathway but not in the CCM pathway without feedback inhibition. Further, α -D-glucose-6P followed the linear path up to the synthesis of the metabolite pyruvate without branching out for the synthesis of any other metabolite in both the aforementioned conditions. Pyruvate acts as a branching point as mentioned earlier. It followed the path through reaction pyruvate \rightarrow OAA \rightarrow phosphoenolpyruvate (PEP) in CCM pathway without feedback inhibition, whereas in the feedback inhibited CCM pathway, it had reaction pyruvate \rightarrow acetaldehyde \rightarrow acetate \rightarrow acetyl CoA. Later, in the feedback inhibited CCM pathway, the reaction path entered into TCA cycle through the reaction acetyl CoA + OAA \rightarrow citrate, which was different from that without feedback inhibition. The metabolite isocitrate had branching and it directly synthesized 2-oxo-glutarate without following the reaction isocitrate \rightarrow oxalosuccinate \rightarrow 2-oxo-glutarate in feedback inhibited CCM pathway. The *S. cerevisiae* optimal CCM pathways under both the aforementioned conditions did not opt for PPP.

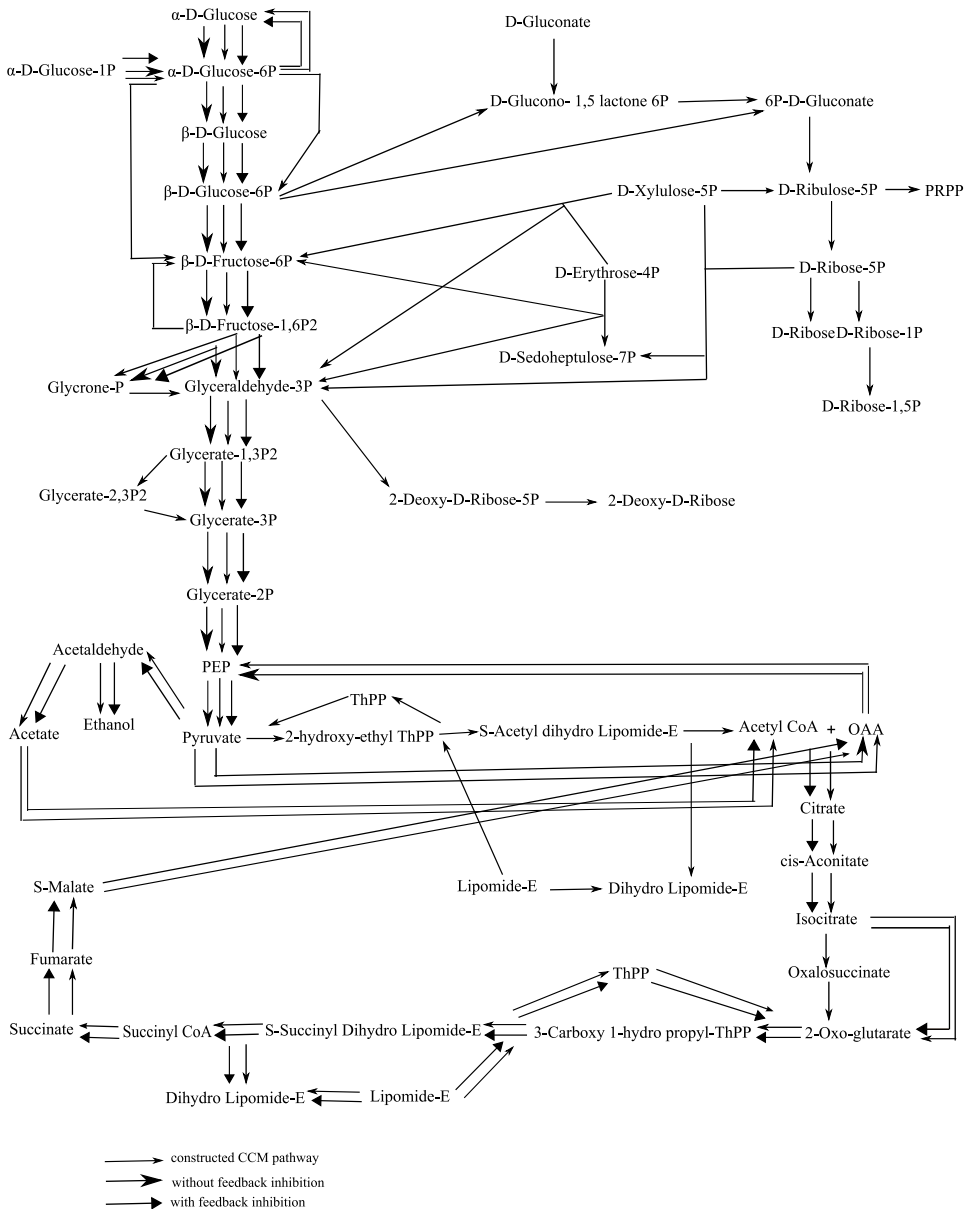


Fig. 4. CCM pathways, with and without feedback inhibition of *S. cerevisiae*. Here optimal pathways obtained by the present method are shown.

4.2.3. Concentration of the regulatory enzymes getting feedback inhibition in *S. cerevisiae* CCM pathway

For inducing the product feedback inhibition, the product itself binds to the corresponding enzyme and thus we obtained lower concentration for the enzymes that

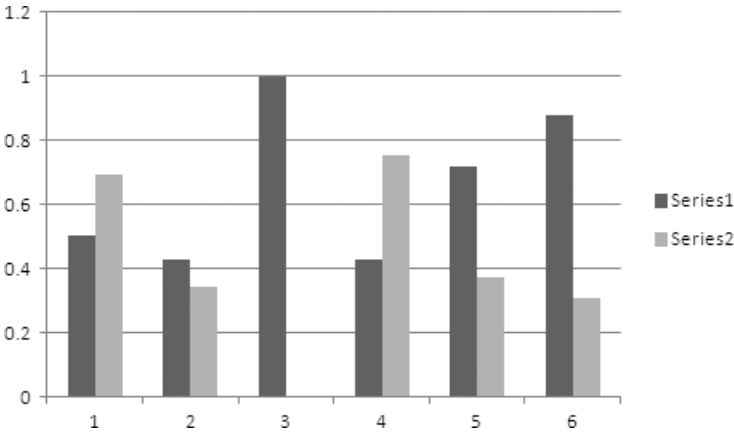


Fig. 5. Graph comparing the concentrations of regulatory enzymes (getting feedback inhibition) with and without feedback inhibition in optimal CCM pathway of *S. cerevisiae*. Series1 shows the concentrations of enzymes present in non-feedback inhibited CCM pathway, and Series2 denotes the concentrations of enzymes present in non-feedback inhibited CCM pathway. Here x -axis denotes the enzymes, where 1: Phosphofructokinase, 2: Pyruvate kinase, 3: Citrate synthase, 4: Isocitrate dehydrogenase, 5: α -ketoglutarate dehydrogenase, 6: Glucose-6-phosphate dehydrogenase, and y -axis shows the c -values (concentrations) of these enzymes.

experience feedback inhibition through their products. We obtained decreased concentrations of the most of these regulatory enzymes. This fact is shown in Table 1 and Fig. 5. The concentration of pyruvate kinase was found to be lower. Another example for which inhibition occurred, is citrate synthase (the regulatory enzyme of TCA cycle), thereby resulting in its lower concentration. An enzyme in TCA cycle, α -ketoglutarate dehydrogenase, which catalyzes the reaction for the synthesis of succinyl-CoA, gets inhibited by its product, hence, its concentration also decreased. The case was similar to glucose-6-phosphate dehydrogenase (the regulatory enzyme of PPP), where its concentration was also decreased due to binding of its product D-Glucono-1,5-lactone-6P.

4.2.4. Concentration of enzymes catalyzing the reactions for the synthesis of considered target products in *S. cerevisiae* CCM pathway with feedback inhibition

Here, we take the case of concentrations of enzymes catalyzing the reactions for the synthesis of the target products of *S. cerevisiae* CCM pathway with feedback inhibition. The concentration of the enzyme, alcohol dehydrogenase, which catalyzes the reaction for the synthesis of ethanol (the target metabolite of glycolysis) was found to be lower (0.911) for the case of feedback inhibition than in non-feedback inhibited optimal CCM pathway (0.9794). In the same manner, we obtained the lower (0.018) concentration of the enzyme, malate dehydrogenase 1 which catalyzes the reaction for the synthesis of OAA (target product of TCA cycle) in the feedback inhibition

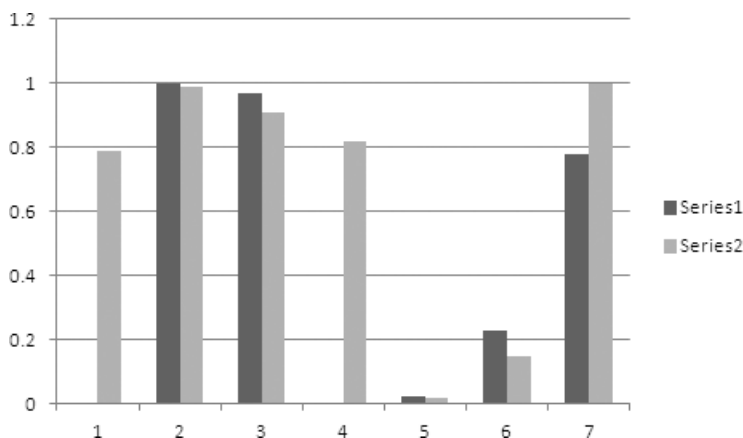


Fig. 6. Graph comparing the concentrations of the enzymes synthesizing the target products directly, with and without feedback inhibited optimal CCM pathways of *S. cerevisiae*. Series1 shows the concentrations of enzymes present in without feedback inhibited CCM pathway, and Series2 denotes the concentrations of enzymes present in feedback inhibited CCM pathway. Here x -axis shows the enzymes where 1: fructose-bisphosphate aldolase, 2: triosephosphate isomerase, 3: alcohol dehydrogenase, 4: pyruvate carboxylase, 5: malate dehydrogenase, 6: transketolase, 7: transaldolase, and y -axis denotes the values of concentrations of these enzymes.

pathway than in without feedback inhibition condition (0.022). The metabolite glyceraldehyde-3P is synthesized from multiple branches in CCM pathway. The concentrations of the enzymes catalyzing the reaction to synthesize glyceraldehyde-3P (an end product of PPP) was also found to be lower in feedback inhibited pathway for many reactions. The corresponding enzymes for the synthesis of glyceraldehyde-3P are fructose-bisphosphate aldolase, triosephosphate isomerase, transaldolase, and transketolase. We observed that concentrations of two of these enzymes became lower *viz.*, triosephosphate isomerase and transketolase (product is glyceraldehyde-3P through PPP). This result is shown in Fig. 6.

4.2.5. Flux distributions of the reactions in *S. cerevisiae* CCM pathway with and without feedback inhibition

We also compared flux values in different branches of CCM pathway with and without feedback inhibition. This comparison is important from a cellular economy level point of view, as cellular resources are limited and there are competing demands among numerous pathways for the same pools of precursors. We hypothesized that the metabolic reactions with increased flux values should be opted by an optimal pathway to increase the production of the target metabolite. The reaction α -D glucose-6P \rightarrow β -D-glucose had higher flux value than the reaction α -D glucose-6P \rightarrow β -D Fructose-6P in without feedback inhibited CCM pathway of *S. cerevisiae*. Likewise, the reaction glycerate-1,3P2 \rightarrow glycerate-3P had higher flux value than the reaction glycerate-1,3P2 \rightarrow glycerate-2,3P2 \rightarrow glycerate-3P in both the conditions.

In feedback inhibited CCM pathway, the reaction acetyl CoA + OAA \rightarrow citrate had higher flux rates than that for the reaction oxaloacetic acid \rightarrow phosphoenol pyruvate.

4.3. Application of the proposed methodology on CCM pathway of *H. sapiens*: With and without feedback inhibition

Here we apply the proposed methodology on CCM pathway of *H. sapiens* with and without feedback inhibition. We considered α -D glucose as the starting metabolite. The metabolites, glyceraldehyde-3P, pyruvate and OAA were considered as target metabolites in CCM pathway of *H. sapiens*. The regulatory enzymes are the same as considered for *S. cerevisiae* feedback inhibited CCM pathway except the enzyme hexokinase. In contrast to *S. cerevisiae*, hexokinase, in *H. sapiens* gets inhibited by its product glucose-6-phosphate.³²

4.3.1. Without feedback inhibition

H. sapiens CCM pathway contained 46 metabolites and 64 reactions (Fig. 7). The combined yield of pyruvate, glyceraldehyde-3P and OAA (target metabolites) was maximized. The concentrations of the enzymes that undergo feedback inhibition are given in Table 1. The combined yield of the target products (z) was 113.0280.

4.3.2. With feedback inhibition

Here the number of metabolites and reactions count to 60 and 85, respectively, if we consider feedback inhibition and enzyme–substrate complexes. We incorporated feedback inhibition of metabolites α -D glucose 6P, β -D-fructose-1,6P2, pyruvate, succinyl-CoA, citrate, 2-oxoglutarate and D-glucono-1,5-lactone-6P. The enzymes being inhibited by the above metabolites and their corresponding concentrations in both the aforesaid conditions are given in Table 1. The combined yield of the target products (z) was found to be 30.3074.

We obtained an optimal pathway from the starting metabolite to the target metabolites as follows. The optimal pathways obtained for *H. sapiens* CCM pathway with and without feedback inhibition are shown in Fig. 7. These optimal paths were found to be different in many aspects. In CCM pathway with and without feedback inhibition, the path from α -D-glucose-6P was- α -D-glucose-6P \rightarrow β -D-glucose-6P. From α -D-glucose-6P, the given CCM pathway has a bifurcation. The CCM pathway without feedback inhibition followed the path β -D-glucose-6P \rightarrow D-glucono-1,5 lactone 6P \rightarrow 6P-D-gluconate \rightarrow D-ribulose-5P \rightarrow PRPP. But in the CCM pathway with feedback inhibition, the path was β -D-glucose-6P \rightarrow β -D-fructose-6P, and then it followed the subsequent reaction. Therefore we can say that for synthesizing the pyruvate (a target product of glycolysis), the optimal path for feedback inhibited CCM pathway differs in many reactions. For example, in without feedback inhibited CCM pathway, the path was glycerate-1,3P2 \rightarrow glycerate-3P, while with feedback inhibition, was glycerate-1,3P2 \rightarrow glycerate-2,3P2 \rightarrow glycerate-3P. Similarly, for

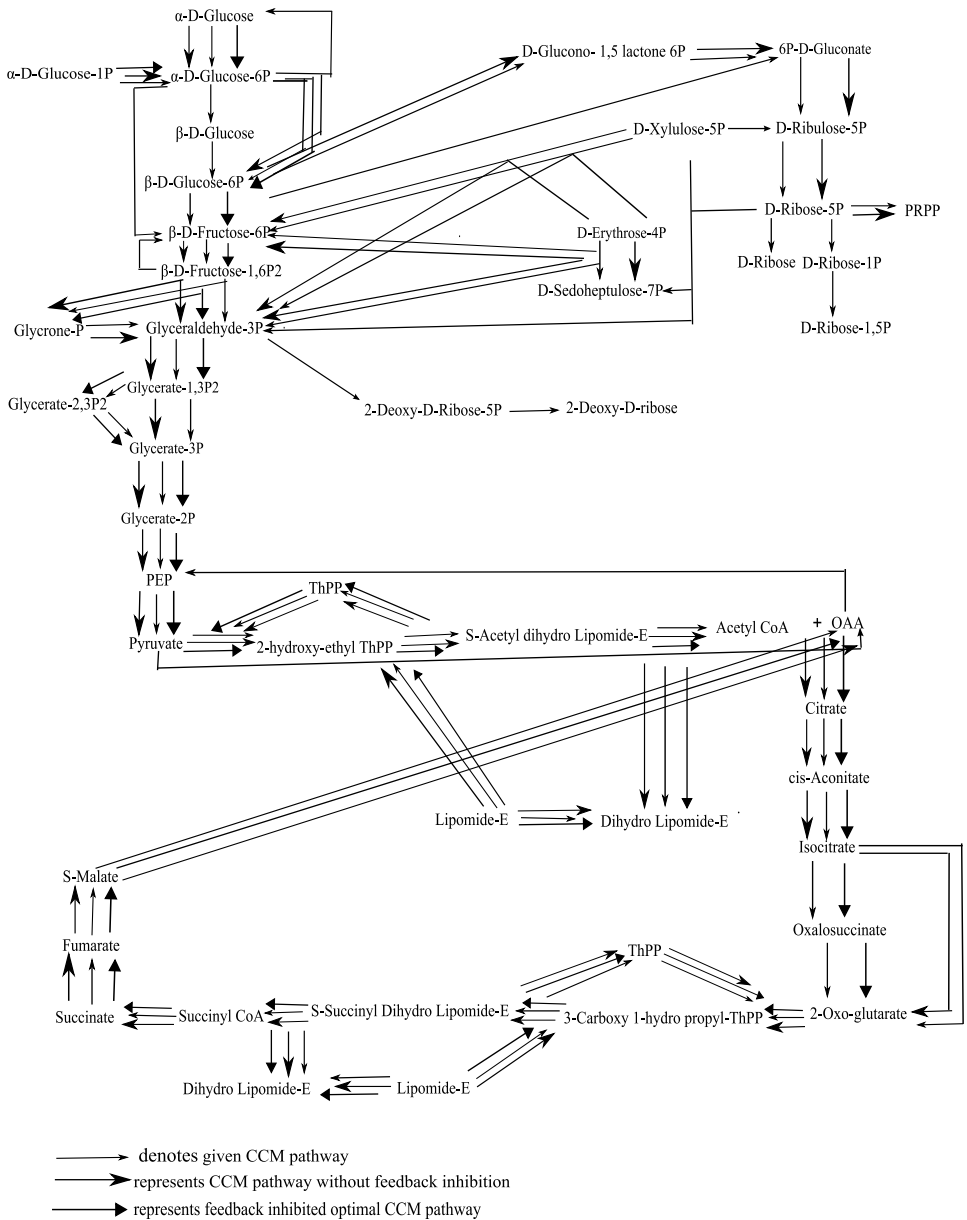


Fig. 7. CCM pathways with and without feedback inhibition, of *H. sapiens*. Here optimal pathways obtained by the present method are shown.

synthesizing OAA (a target product of TCA), isocitrate \rightarrow 2-oxoglutarate was the path followed by the feedback inhibition pathway. But in feedback inhibited optimal CCM pathway, it had an intermediate substrate also, the reaction was isocitrate \rightarrow oxalosuccinate \rightarrow 2-oxoglutarate.

In the optimal CCM pathway without feedback inhibition, glyceraldehyde-3P (a target product of PPP) was formed by β -D-fructose-1,6P2, D-xylulose-5P and D-erythrose-4P. D-erythrose-4P also gave glyceraldehyde-3P through combining with β -D-fructose-6P. While in CCM pathway with feedback inhibition, it was formed by only one reaction *i.e.*, β -D-fructose-1,6P2 through a different reaction. In this way, the path for final product differed in the conditions with and without feedback inhibited CCM pathway. Moreover, it is interesting to note that the optimal path followed by feedback inhibited CCM pathway was found to be shorter than the other case.

4.3.3. Concentration of the regulatory enzymes getting feedback inhibition in *H. sapiens* CCM pathway

Flux of a reaction is controlled by the concentration of the enzyme catalyzing the reaction. The concentration of certain enzymes catalyzing the reactions to yield a particular target metabolite should be low when the target metabolite is not needed by the cell. If the concentration of such an enzyme is high, a resulting metabolite in a path will bind to the enzyme's allosteric site and change its conformation, which prevents the binding of the substrate with the enzyme. Thus the effective enzyme concentration gets lowered. This fact is depicted in Table 1 and Fig. 8. We obtained

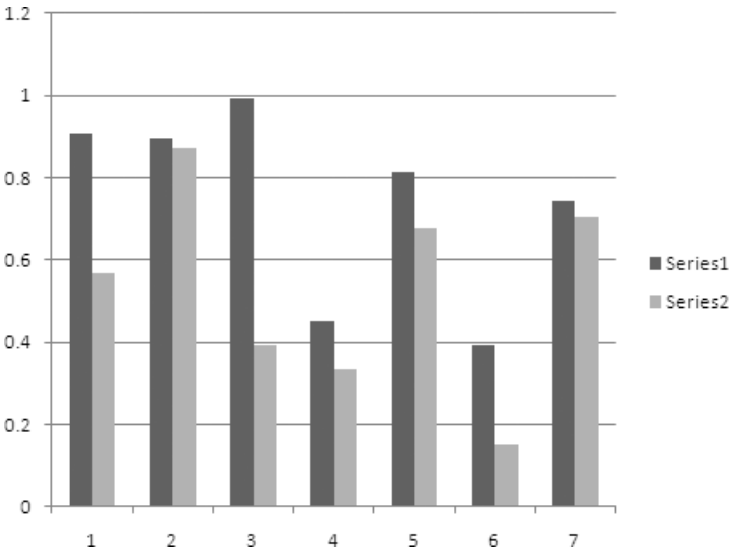


Fig. 8. Graph comparing the concentrations of the regulatory enzymes (getting feedback inhibition) with and without feedback inhibited optimal CCM pathway of *H. sapiens*. Series1 shows the concentrations of enzymes present without feedback inhibited CCM pathway, and Series2 denotes the concentrations of enzymes present in feedback inhibited CCM pathway. Here *x*-axis denotes the enzymes where 1: Hexokinase, 2: Phosphofructokinase, 3: Pyruvate kinase, 4: α -ketoglutarate dehydrogenase, 5: Citrate synthase, 6: Isocitrate dehydrogenase, 7: Glucose-6-phosphate dehydrogenase, and *y*-axis shows the values of concentrations of these enzymes.

a decreased concentration value for hexokinase due to its inhibition by α -D glucose-6P through binding. We observed that the concentration of the phosphofruktokinase was lower in feedback inhibited CCM pathway, as the cell did not need an excess amount of metabolite β -D-fructose-1,6P2. A similar kind of observation was found for the metabolite pyruvate, which is the end product of glycolysis. The concentration of its corresponding enzyme pyruvate kinase became lower as the cell's metabolic system does not need pyruvate in excess. Another example for which inhibition occurred was citrate (a metabolite in TCA cycle). The concentration of its catalyzing enzyme citrate synthase became lower and thus it ceased the further synthesis of citrate. The lower concentration of isocitrate dehydrogenase in the feedback inhibited pathway was observed, where the enzyme was inhibited by 2-oxoglutarate. Another enzyme in TCA cycle is α -ketoglutarate dehydrogenase, which catalyzes the reaction for the synthesis of succinyl-CoA and gets inhibited by the product. Its concentration also became lower in feedback inhibited pathway. The case was similar with glucose-6-phosphate dehydrogenase (the regulatory enzyme of PPP), where its concentration was also found to be decreased in feedback inhibited CCM pathway.

4.3.4. Concentration of enzymes catalyzing the reactions for the synthesis of considered target products in *H. sapiens* CCM pathway with feedback inhibition

Here we take the case of concentrations of enzymes catalyzing the reactions for the synthesis of the target products of *H. sapiens* CCM pathway. First, we observed that the concentration of the enzyme pyruvate kinase, which catalyzes the reaction for the synthesis of pyruvate (the target metabolite of glycolysis), was found to be lower (0.39084) in feedback inhibited CCM pathway than without feedback inhibited pathway (0.99406). Similarly, the concentration of the enzyme, malate dehydrogenase 1, which catalyzes the reaction for the synthesis of OAA (target product of TCA cycle), had been found to be lower (0.0066929) in feedback inhibited pathway than in without feedback inhibition condition (0.60412). This observation may be due to the fact that the product concentration is kept in control according to cell's requirement in feedback inhibited pathway. The concentrations of the enzymes catalyzing the reaction to synthesize glyceraldehyde-3P (an end product of PPP) was also found to be lower in feedback inhibited pathway in many reactions. The metabolite glyceraldehyde-3P is synthesized from multiple branches in CCM pathway. The corresponding enzymes for the synthesis of glyceraldehyde-3P are aldolase A, triosephosphate isomerase 1, transaldolase 1, and transketolase. We observed in our study that its corresponding enzymes' concentrations became lower (at three reactions) *viz.*, transaldolase 1 and transketolase (product is glyceraldehyde-3P through PPP), when the pathway no longer demanded for the product. This result is shown in Fig. 9. In this way, the cell's metabolic system keeps checking on the concentration of its several metabolites. It has been found that no single enzyme, on being inhibited, exists within a

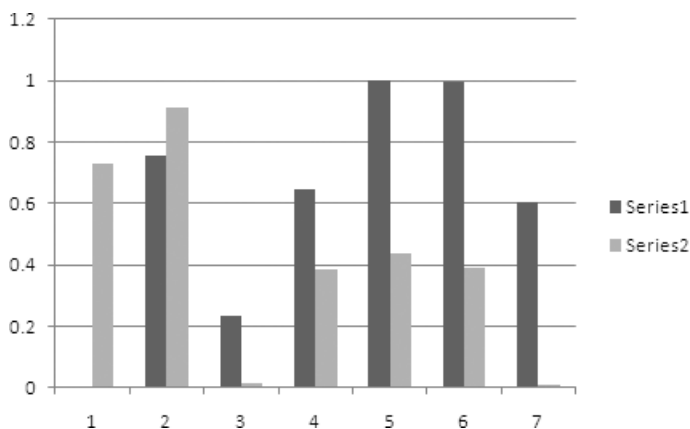


Fig. 9. Graph comparing the concentrations of the enzymes synthesizing the target products directly, with and without feedback inhibited optimal CCM pathways of *H. sapiens*. Series1 shows the concentrations of enzymes present without feedback inhibited CCM pathway and Series2 denotes the concentrations of enzymes present in feedback inhibited CCM pathway. Here x -axis shows the enzymes where 1: Aldolase A, fructose-bisphosphate, 2: Triosephosphate isomerase 1, 3: Transketolase, 4: Transaldolase 1, 5: Transketolase, 6: Pyruvate kinase, 7: Malate dehydrogenase 1, and y -axis denotes the values of concentrations of these enzymes.

pathway, but rather the activities of several enzymes in a pathway change in tandem in response to changing conditions and the ensuing metabolic signals.

4.3.5. Flux distributions of the reactions in optimal CCM pathways of *H. sapiens*: With and without feedback inhibition

Here we compare flux values in different branches of the CCM pathway with and without feedback inhibition. This comparison is important from a cellular economy level point of view, as cellular resources are limited and there are competing demands among numerous pathways for the same pools of precursors. We hypothesized that the metabolic reactions with increased flux values should be opted by an optimal pathway to increase the production of the target metabolite. For instance, the reaction α -D glucose-6P \rightarrow β -D-glucose-6P gave higher flux value than that of α -D glucose-6P \rightarrow β -D-glucose \rightarrow β -D-glucose-6P in CCM optimal pathway with and without feedback inhibition (Fig. 10). Another example in an optimal pathway without feedback inhibition condition is the reaction forming 6P-D-gluconate. This reaction (β -D-glucose-6P \rightarrow D-glucono-1,5 lactone 6P \rightarrow 6P-D-gluconate) had a higher flux value than that of the reaction β -D-glucose-6P \rightarrow 6P-D-gluconate. A similar kind of observation was found for the reaction glycerate-1,3P2 \rightarrow glycerate-3P, which had higher flux value than that without feedback inhibition. The reverse was true for an optimal feedback inhibited CCM pathway *i.e.*, the reaction glycerate-1,3P2 \rightarrow glycerate-2,3P2 \rightarrow glycerate-3P. We had shown aforesaid flux comparison in both the studied conditions in Figs. 10 and 11.

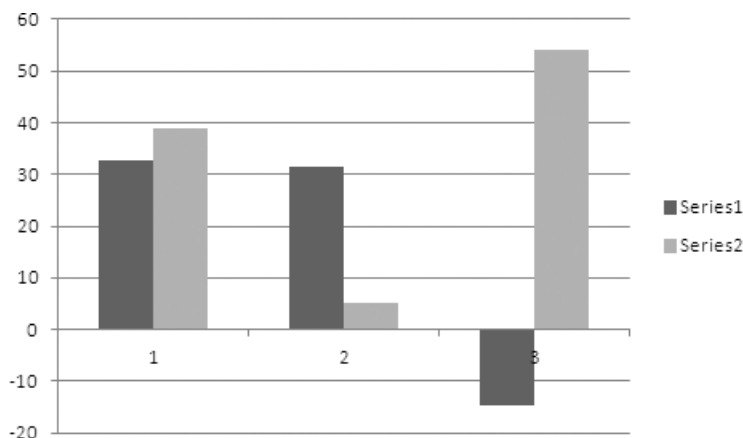


Fig. 10. Graph comparing the flux values in different branches of optimal CCM pathway of *H. sapiens* without feedback inhibition. In the graph, x -axis refers to metabolic reactions *viz.*, 1, Series1: α -D glucose-6P \rightarrow β -D-glucose, 1, Series2: α -D glucose-6P \rightarrow β -D-glucose-6P, 2, Series1: β -D-glucose-6P \rightarrow D-glucono-1,5 lactone 6P, 2, Series2: β -D-glucose-6P \rightarrow 6P-D-gluconate, 3, Series1: glycerate-1,3P2 \rightarrow glycerate-2,3P2, 3, Series2: glycerate-1,3P2 \rightarrow glycerate-3P, and y -axis denotes the flux values.

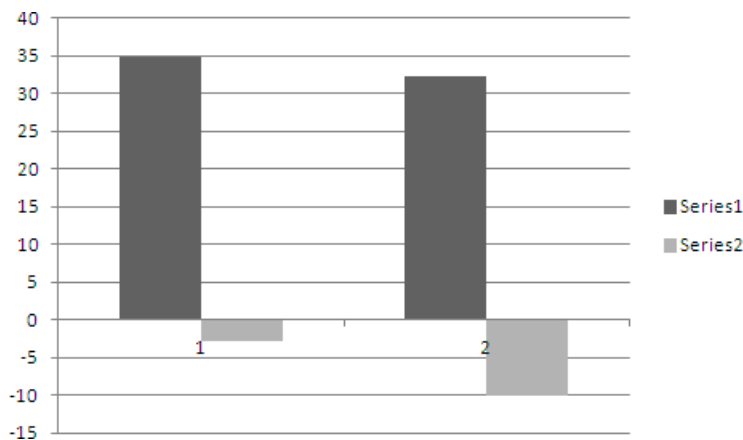


Fig. 11. Graph comparing the flux values in different branches of optimal CCM pathway of *H. sapiens* with feedback inhibition. In the graph, x -axis refers to metabolic reactions *viz.*, 1, Series1: α -D glucose-6P \rightarrow β -D-glucose-6P, 1, Series2: α -D glucose-6P \rightarrow β -D-glucose, 2, Series1: glycerate-1,3P2 \rightarrow glycerate-2,3P2, 2, Series2: glycerate-1,3P2 \rightarrow glycerate-3P, and y -axis denotes the flux values.

4.4. Comparison between the optimal CCM pathways of *S. cerevisiae* and *H. sapiens*: With and without feedback inhibition conditions

We opted for two different organisms *viz.*, *S. cerevisiae* and *H. sapiens* to observe the effect of feedback inhibition in their CCM pathways. *Saccharomyces cerevisiae*

is a kind of single cell yeast under the kingdom fungi. It is considered as a model eukaryotic organism. As an eukaryote, a majority of the yeast genes and proteins have human homologs,³³ thus it would help us to understand the human genome. Since the complete genome sequence of *S. cerevisiae* is now available, mutants unique to eukaryotic organisms can now be expressed in an eukaryote as opposed to study of similar gene expression in prokaryotes. Another advantage is its fast growth rate.³⁴ In contrast to *S. cerevisiae*, a single cell eukaryote, *H. sapiens* is an eukaryote that possesses complex organ systems.

Here we describe the differences between the optimal CCM pathways of *S. cerevisiae* and *H. sapiens*, with and without feedback inhibition conditions. One of the major differences between the CCM pathways of these two organisms is in their construction: *S. cerevisiae* synthesizes ethanol in aerobic and anaerobic conditions in high glucose environment, whereas in *H. sapiens*, pyruvate is formed as target metabolite. The major differences among reactions present in optimal CCM pathways of *S. cerevisiae* and *H. sapiens*, with and without feedback inhibition conditions, are as follows.

- (1) Optimal CCM pathway for *S. cerevisiae* in without feedback inhibition condition followed a direct route from the metabolite α -D-glucose to synthesize pyruvate, whereas in optimal CCM pathway for *H. sapiens* without feedback inhibition condition, it had branched out at the metabolite α -D-glucose-6P and then followed the reaction α -D-glucose-6P \rightarrow β -D-glucose-6P. These routes were in accordance with feedback inhibition condition also.
- (2) The second branching point is at the metabolite glycerate-1,3P2, where the reaction glycerate-1,3P2 \rightarrow glycerate-3P was similar in both the organisms, without feedback inhibition. However, this branching point found to be different in optimal CCM pathways with feedback inhibition for *S. cerevisiae* and *H. sapiens*. In *S. cerevisiae*, the reaction was glycerate-1,3P2 \rightarrow glycerate-3P, whereas it followed the path through the reaction glycerate-1,3P2 \rightarrow glycerate-2,3P2 \rightarrow glycerate-3P in *H. sapiens*.
- (3) Another branching point was at pyruvate, which directly synthesized OAA through the enzyme pyruvate carboxylase in the optimal CCM pathway of *S. cerevisiae* without feedback inhibition condition, whereas OAA was not synthesized directly in optimal CCM pathway of *H. sapiens* with and without feedback inhibition conditions. OAA also synthesized PEP in *S. cerevisiae* without feedback inhibition condition but not in the CCM pathway of *H. sapiens*. OAA did not enter into the TCA cycle in optimal CCM pathway of *S. cerevisiae* without feedback inhibition condition, whereas it followed the reactions of TCA cycle in optimal CCM pathways *H. sapiens* in both the conditions.
- (4) Another aspect of variation in optimal CCM pathways of both these organisms was the presence of the reactions that enter into PPP. The optimal CCM pathway of *S. cerevisiae* did not follow the reactions for PPP in both the aforesaid conditions. However, the optimal CCM pathway for *H. sapiens*,

without feedback inhibition condition, had reactions that entered into PPP and synthesized glyceraldehyde-3P along with other metabolites.

4.5. Comparison of the results obtained for the optimal CCM pathways of *H. sapiens* with EPA

Extreme pathways are mathematically derived vectors that describe the conical steady state solution space for flux distributions of a metabolic network.³⁰ Thus extreme pathways are used to describe a linear set of reactions linking substrate to product, and to characterize the relative flux levels through all the reactions necessary to convert substrates to products.³⁵ The set of extreme pathways is actually a set of all possible steady-state fluxes that map a metabolic network and can be computed from the stoichiometric matrix. These pathways represent the edges of the steady-state flux cone derived from convex analysis, and thus they are being used to represent flux distribution of a metabolic network. These pathways can be used to interpret metabolic functioning and control; thus we have performed a comparative analysis of the results obtained by our method with the EPA.

To find out extreme pathways for the CCM pathway (for both with feedback and without feedback inhibition conditions), we used ExPA program.^{19,20} We found 98 extreme pathways for CCM pathway without feedback inhibition. Some of them were combined to get a pathway as shown in Fig. 12. These extreme pathways consisted of combination of one or more reactions of CCM pathway.

Similarly, we found 109 extreme pathways for CCM pathway with feedback inhibition. As in the previous case, we had a pathway as shown in Fig. 12, by combining some of the extreme pathways. These results gave many missing links. For example, the reaction, α -D glucose \rightarrow α -D glucose 6P was missing in the case of CCM pathway without feedback inhibition (Fig. 12). Similarly, in the case of feedback inhibition, the reactions, α -D glucose \rightarrow α -D glucose 6P and β -D glucose \rightarrow β -D glucose 6P were present but the intermediate link *i.e.*, α -D glucose 6P \rightarrow β -D glucose, as shown in Fig. 13, was absent. It is to be mentioned here that EPA does not consider enzyme concentrations catalyzing the metabolic reactions in contrary to the present method.

4.6. Possible biological validation

Here we provide the biological validation for the results obtained for optimal CCM pathways of *S. cerevisiae* and *H. sapiens* under both of the aforementioned conditions.

4.6.1. For CCM pathway of *S. cerevisiae*

Here we validate some of the results obtained for CCM pathways of *S. cerevisiae* through comparing them with existing lab experiments.

We compared different enzyme concentrations, flux rates of different branches of the CCM pathway under both aforesaid conditions *viz.*, with and without feedback

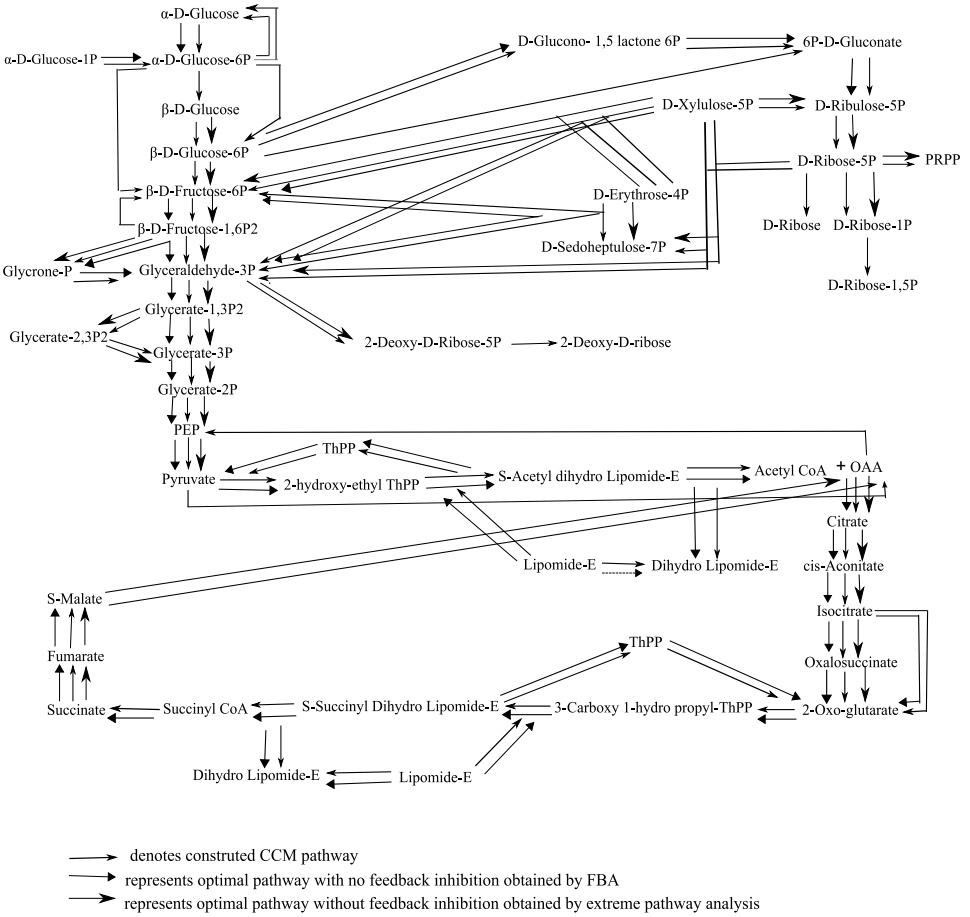


Fig. 12. Comparison of the optimal CCM pathway of *H. sapiens*, without feedback inhibition, obtained by the present method.

inhibition. Our assumption was that the concentration of an enzyme (the regulatory one) catalyzing the reaction to synthesize a particular metabolite would be lower when it is not needed by the cell's system (in feedback inhibited pathway). The concentration of the enzyme that synthesizes the target product was also assumed to be lower in the feedback inhibited pathway as it is undesirable to accumulate the target metabolite in excess, thus we can say that feedback inhibition condition lessens the enzyme excess capacity. We have also hypothesized that the reactions present in an optimal pathway would have higher flux rates.

The *in vivo* activity pattern was determined for the reaction catalyzed by PEP carboxykinase, and found that it was only detected at pH values 7.0 and 7.5.³⁶ We found a moderate concentration (0.53485) of enzyme PEP carboxykinase in optimal CCM pathway of *S. cerevisiae* under feedback inhibition and very low concentration

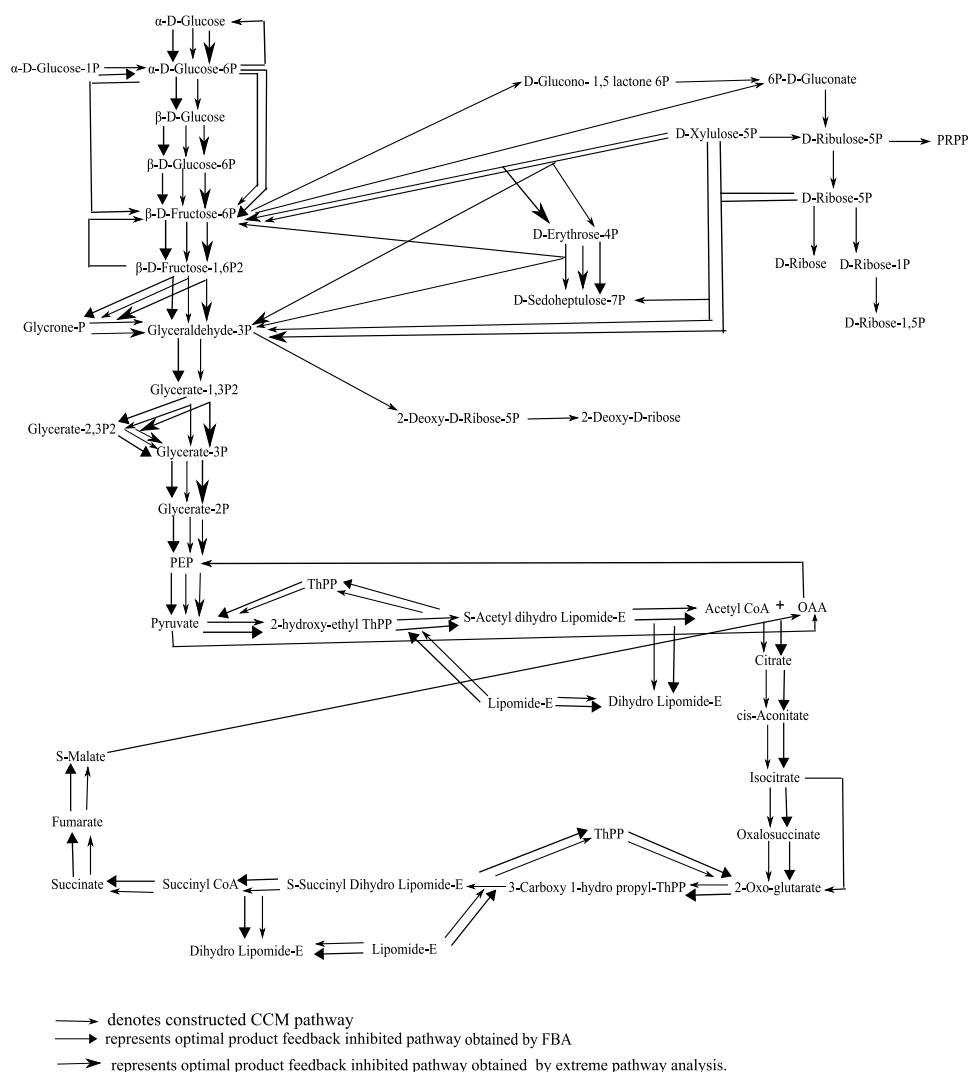


Fig. 13. Comparison of the optimal CCM pathway of *H. sapiens*, with feedback inhibition, obtained by the present method.

(0.04556) in non-feedback inhibited optimal CCM pathway. The authors also found very low TCA cycle and PPP activities through METAFoR analysis by GC-MS of *S. cerevisiae* in glucose batch culture. Moreover, low TCA cycle activity and concomitant ethanol production were also expected for the respiro-fermentative *S. cerevisiae*.³⁷ This is in accordance with our modeling results in a way as we also found that the optimal CCM pathways under both aforesaid conditions does not follow the reactions for PPP. Moreover, optimal CCM pathway without feedback

inhibition did not enter TCA cycle, rather it synthesizes PEP through substrate OAA and enzyme PEP carboxykinase.

A study shows that PPP serves for biosynthesis only *i.e.*, phosphoenolpyruvate (PEP) is entirely generated via glycolysis.³⁸ Gancedo *et al.*³⁹ also estimated that *S. cerevisiae* catabolizes less than 1% of the glucose via PPP to pyruvate. Moreover, the majority of the cytosolic OAA was synthesized via anaplerotic carboxylation of pyruvate.³⁸ We also found that OAA is synthesized directly through pyruvate in optimal CCM pathway without feedback inhibition.

A study done by Teusink *et al.*,⁴⁰ monitored glucose consumption and product formation for 45–60 min in yeast and found that the most of the glucose is converted into ethanol and CO₂. Mcghee *et al.*⁴¹ did a similar kind of study and investigated the effect of different cell ages on the conversion of glucose to ethanol by *S. cerevisiae* NRRL Y-2034. They found that 100% of the available glucose had been converted to ethanol in approximately 3 months by the 96-h-old yeast cells in the continuous flow fermentor. We also found the path for ethanol production through glucose in optimal CCM pathway with feedback inhibition (Fig. 4). Pyruvate is a key intermediate in sugar metabolism. As described earlier, pyruvate metabolism can occur through three different pathways.²⁴ During fermentative growth, pyruvate is decarboxylated into acetaldehyde by pyruvate decarboxylase, which later reduced to ethanol in the cytosol by alcohol dehydrogenase1.⁴² Moreover, a study suggest that pyruvate was first decarboxylated in the cytosol and that the acetaldehyde was oxidized to acetate in the mitochondria.⁴³ We obtained got the path in optimal CCM pathway with feedback inhibition through which enzyme pyruvate decarboxylase (0.66061) catalyzes the reaction to synthesis acetaldehyde from pyruvate that later forms acetate.

The metabolic shift from oxidative to fermentative growth was studied, which was observed through the complex changes of carbon flux throughout the CCM in *S. cerevisiae*.⁴⁴ This has involved a redirection of flux from TCA cycle toward the fermentative pathway and a substantial decrease of the relative flux through PPP. Moreover, increased flux rate had been observed through pyruvate carboxylase and the highest flux through pyruvate decarboxylase was observed at the maximum production of ethanol and acetate. They obtained a high production of acetaldehyde for all physiological states, which was later utilized for the synthesis of acetyl CoA under purely oxidative growth. Although this particular study was based on metabolic fluxes comparison and in contrast to this, we compared enzyme concentrations of various metabolic reactions present in CCM pathway in the present study, yet we obtained the similar kind of results in optimal CCM pathways. For example, we obtained OAA from pyruvate (reaction catalyzed by the enzyme pyruvate carboxylase) in non-feedback inhibited optimal CCM pathway. Another similarity lies in the production of acetaldehyde from the metabolite pyruvate that synthesizes acetate, and later forms acetyl CoA in feedback inhibited optimal CCM pathway.

It had been observed that the disruption of the gene *zwf1*, encoding glucose-6-phosphate dehydrogenase (the first enzyme in PPP) in *S. cerevisiae* did not affect

the cell growth in rich media.⁴⁵ This had led to the study of alternative routes for NADPH synthesis. In this regard, the authors found that the reaction catalyzed by cytosolic acetaldehyde dehydrogenase6 (enzyme Ald6 for catalyzing reaction acet-aldehyde \rightarrow acetate) is an important source of reducing equivalents. In this regard, we also obtained similar result in feedback inhibited optimal CCM pathway, where acetaldehyde dehydrogenase (0.24084) synthesized acetate from acetaldehyde.

The critical role of pyruvate decarboxylase and alcohol dehydrogenase in determining the rate of ethanol production was confirmed by using pyruvate decarboxylase constitutive mutants.⁴⁶ By deriving strains with altered levels of these two enzymes, it had been found that higher levels of the enzymes pyruvate decarboxylase and alcohol dehydrogenase were necessary for faster ethanol production. This is in accordance with the path, we got in optimal CCM pathway with feedback inhibition, where it had the reactions that lead to ethanol production *viz.*, pyruvate \rightarrow acet-aldehyde \rightarrow ethanol.

4.6.2. For CCM pathway of *H. sapiens*

Here we try to validate some of the results obtained by the present method. The validation is done by showing resemblance of the present results with some earlier investigations. We could not find any direct evidence to show the above fact, hence we mention here a different but related observations.

Figure 7 shows the optimal CCM pathways of *H. sapiens* obtained for with and without feedback inhibition, by the method, through which the amount of the target metabolites, grown on a starting substrate (α -D-glucose) is maximum. In the case of feedback inhibited CCM pathway, the number (37) of the reaction in the optimal pathways had been found to be less than that (41) for the case of CCM pathway without feedback inhibition. The total concentrations of the target metabolites was more ($z = 113.0280$) under without feedback inhibition than that ($z = 30.3074$) in the other case. These facts are mentioned in Secs. 4.3.1 and 4.3.2. That is, we may assume that the concentration (z) of the target products decreases with the decrease in the number of reactions from the starting metabolites to the targets. This result is in accordance with the investigation of Feng *et al.*,⁴⁷ where the yield of biosynthetic products had been shown to decrease exponentially with the number of steps away from central metabolism in *S. cerevisiae*. Yeast has a high level of conservation between its cellular processes and those of mammalian cells along with advantages such as simple growth requirements, rapid cell division, ease of genetic manipulation and well-characterized genome-wide analysis of biological functions. Thus, it is widely used as a model organism for investigating many aspects of eukaryotic cell biology.⁴⁸

We integrated glycolysis pathway, TCA cycle and PPP to form the CCM pathway, and one can find common metabolites in these three pathways. The glycolytic/gluconeogenic cycle intersects with the glycogenesis/glycogenolysis cycle, TCA and PPP, being thus embedded in a larger carbon metabolic network. We also mentioned

earlier that the interchange of carbon atoms between glycolysis pathway and PPP takes place at the level of fructose-6-phosphate and/or glyceraldehyde-3-phosphate. An optimal pathway under no feedback inhibition, followed the reaction β -D-glucose-6P \rightarrow D-glucono-1,5 lactone 6P. Marin *et al.*⁴⁹ used mass isotopomer distribution analysis (MIDA) using GC coupled to MS (GCMS) for providing a metabolic profile of the individual fluxes in response to several substrate inputs using [1,2-¹³C₂] glucose in hepatocytes. The metabolism of [1,2-¹³C₂]glucose resulted in the rearrangement, exchange, or loss of the ¹³C label. The recycling of ¹³C from TCA and phosphoenolpyruvate typically led to almost symmetrically labeled glucose molecules. Thus the lack of symmetry of the ¹³C distribution in glycogen indicated that the formation of M1 glucose (molar fractions) was primarily by the action of glucose-6-phosphate dehydrogenase (G6PD) *i.e.*, the recycling of ¹³C from PPP. These results support the systems biology notion that network analysis provides an integrated view of the physiological state.

Interestingly, we found increased concentration of glucokinase (GK) in feedback inhibited CCM pathway in comparison with pathway without feedback inhibition. This is an example of an indirect evidence of stimulatory effects of fructose on GK. Glucokinase is also present in the pancreatic β -cell, thus the stimulatory effects of fructose and glyceraldehyde on glucose phosphorylation also occur in the endocrine cell. The phosphorylation of glucose was measured by Schaftingen *et al.*⁵⁰ in the suspensions of freshly isolated rat hepatocytes. They found that fructose (0.2 mM) stimulated the rate of phosphorylation of 5 mM glucose by 2–4 fold, although this observation could not be found for 40 mM glucose as fructose stimulates GK in the liver cell.

In our study, we monitored the concentrations of CCM enzymes and found that they varied in their respective concentrations under with feedback and without feedback inhibition. For example, phosphoglucomutase (PGM) (α -D-glucose-1P \rightarrow α -D-glucose-6P) concentration was higher in the CCM pathway without feedback inhibition. But the concentrations in both the aforesaid conditions had very less difference between them. Both aldolase A (ALD)(β -D fructose-1,6P2 \rightarrow glyceraldehyde-3P) and phosphoglycerate kinase 1 (PGK) (glycerate-1,3P2 \rightarrow glycerate-3P) concentrations in the feedback inhibited CCM pathway increased, while glucose-6-phosphate isomerase/phosphoglucoisomerase (PGI) (α -D-glucose-6P \rightarrow β -D-glucose-6P), enolase 1 (ENOL) (glycerate-2P \rightarrow phosphoenolpyruvate) and pyruvate kinase (PK) (phosphoenolpyruvate \rightarrow pyruvate) concentration decreased in the same pathway. Triosephosphate isomerase 1 (TPI) (glycerone-P \rightarrow glyceraldehyde-3P) concentration had been found lower in both the aforesaid cases. Guppy *et al.*⁵¹ found that the capacity of the glycolytic pathway in white muscle was drastically upregulated compared to the pathway in red muscle of *Tuna*. It occurs through evolution within specific lineages and through ontogeny within species. To achieve this large-scale upregulation, the enzymes for each individual step are changed to a variable degree. For example, they found that hexokinase and phosphofructokinase changed the least, while TPI and lactate dehydrogenase (LDH) increased the most. Glycogen

phosphorylase (PHOS), PGM, PGI, ALD, PGK, ENOL and PK in *tuna* white muscle was elevated by an intermediate amount and were expressed at ~ 35 -fold higher levels than in red muscle.

5. Discussion

It has become a well established fact now in the field of enzymology that one of the regulatory mechanisms through which an organism controls its biosynthetic pathway is by feedback inhibition. This control mechanism adjusts the rate of production of the end products of a pathway.⁵² The organism has the advantage of feedback inhibition phenomenon as it avoids wasting of resources. Certainly it is a widespread and effective metabolic control. Many biosynthetic pathways in bacteria (like, *E. coli*) have feedback inhibition, and a few instances are also known for higher organisms. For example, *E. coli* grows normally with the supply of isoleucine (amino acid) that blocks the activity of enzyme taking part in step one. This occurs as isoleucine combines with threonine deaminase enzyme. This inhibition differs from repression. Enzyme does not function in feedback inhibition, while mRNA transcription ceases in repression activity, and hence enzyme does not get synthesized.

We have considered glycolysis pathway, TCA cycle and PPP as a part of CCM pathway for *S. cerevisiae* and *H. sapiens* and implemented FBA with required modifications as described in Sec. 3. We have found that the FBA implementation gives better results as it consists of all the reactions from CCM pathway in parallel while the EPA output gives individual reactions in a sequential manner. Extreme pathway gives a set of individual reaction or the subset of individual reactions which are considered as extreme pathways. The major drawback with EPA is the absence of enzyme concentrations in its formulation. The modified methodology can be applied in metabolic engineering and biotechnology industry as it helps to opt for an optimal pathway with minimal wastage of cell's resources. It also helps in identifying the corresponding gene which can be activated later to express the enzyme in the required concentration.

There are some contradictory facts available. The control of glycolysis by energetic status can be exerted by changes in ADP inhibition of ATPase(s). This control would work through allosteric activation of phosphofructokinase. ATPase activation would increase ADP, it activates PGK and PK and cause consequent decrease in 1,3-bisphosphoglycerate and phosphoenolpyruvate, respectively. The reduced product inhibition of glyceraldehyde-3-phosphate dehydrogenase and enolase would be transmitted backwards up the glycolytic chain, which accounts for the negative flux/metabolite co-responses have been observed.⁵³

We have obtained the decreased level of phosphofructokinase (β -D fructose-6P \rightarrow β -D fructose-1,6P2) in both kinds of aforesaid cases and the lower and higher flux rates in *H. sapiens* without and with feedback CCM pathway, respectively. In the case of yeast, where signal from fructose-2,6P2 was abolished by blocking its synthesis. It has an affect on metabolite concentrations, but not on glycolytic flux.⁵⁴

Even, the overexpression of phosphofructo-2-kinase does not effect the flux in yeast cells with elevated levels of fructose-2,6P2.⁵⁵ There are significant progresses in our understanding of the underlying mechanisms of these metabolic pathways and the potential therapeutic implications in metabolic disorders. It is an already known that if enzyme does not regulate certain reactions in the metabolic pathway, the product starts accumulating in the cell, resulting in disease. For example, hemolytic anemia is associated with hexokinase, pyruvate kinase and glucose-6-phosphate dehydrogenase (G6PDH) deficiency.^{56–58} Increased aerobic glycolysis⁵⁹ is commonly seen in a wide spectrum of human cancers, and hypoxia is present in most tumor microenvironment. Thus novel glycolytic inhibitors need likely to be developed as a new class of anticancer agents.⁶⁰

6. Conclusion

Even if a sufficient amount of the substrate is present in the pathway, the concentration of the product metabolite may be found lower as the rate of production of the target metabolite also depends on the concentration of catalyzing enzyme. There are evidences depicting that cell has the capacity to synthesize close to the maximum amount of biomass per unit of nutrient consumed.¹² This observation is the motivation for us to study the regulatory mechanism of the metabolic pathways which gives them such optimality.

In the present article, we have developed a methodology to find an optimal pathway that a cell may likely to opt for, with and without feedback inhibition for CCM pathways of *S. cerevisiae* and *H. sapiens*. We have used two different formulations for two different kinds of enzymes present in CCM pathway *i.e.*, the enzymes catalyzing the metabolic reactions, and those responsible for both catalyzing the reactions as well as regulating the metabolism in the system. Thus, we have got two different optimal pathways to study both the feedback and without feedback inhibition conditions. We have applied the formulation introduced by De *et al.*¹⁸ for the metabolic pathway without feedback inhibition. We have introduced a new formulation to identify an optimal pathway with feedback inhibition in CCM pathways of both of aforementioned organisms. These formulations are in terms of the level of enzyme concentration, which yields the maximum rate of production of a metabolite starting from a given substrate under two conditions *viz.*, with and without feedback inhibition in CCM pathway. We have identified the reactions with lower enzyme concentrations to make an optimal pathway in both the aforementioned conditions.

We have compared the results obtained for optimal CCM pathways of *S. cerevisiae* and *H. sapiens* with and without feedback inhibition conditions. We have also compared the optimal CCM pathways of *H. sapiens* in both aforementioned conditions obtained by FBA with EPA, which does not consider enzyme concentrations and finds the flux vectors through optimization and gives individual metabolic reactions as output. On the other hand, the present method generates a possible flux

vectors and, then finds an optimal pathway and considers all the reactions in parallel. Some of the results have been verified through earlier studies.

Constructing CCM pathway involves glycolysis pathway, TCA cycle and PPP, which have few nodes in common. These common nodes join these three separate pathways and forms the CCM pathway. This kind of phenomenon is found in pathway cross talk, where a molecular species (forming a common node) of one pathway is reused in another pathway. This leads to an integrated study of two/more metabolic pathways simultaneously.

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