A Bi-level Optimisation Approach for the Productivity and Thermodynamic Performance of Metabolic Systems

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Abstract

A metabolic network is most often interpreted and modeled in terms of a collection of enzyme-catalyzed reactions with transport phenomena that utilise substrate metabolites to generate final metabolites. The complexity of the metabolic reaction systems necessitates the development of the integrated approaches to analyse and interpret the systemic properties of cellular metabolism, which shifts the emphasis from single metabolic reactions to systemic pathways defined by elementary flux mode (EFM) analysis. In this work a methodology is developed to establish a rational metabolic engineering strategy for the elucidation and optimisation of metabolic systems, by combining the metabolic flux analysis (MFA) and pathway identification with the thermodynamic analysis of the metabolic system. A bi-level optimisation strategy has been developed to predict the optimal pathways for the maximum productivity and the operating conditions / performance in the first and the second level respectively, for achieving the desired objectives. In the first level, a systematic enumeration of pathways is described by the elementary flux mode (EFM) analysis, which provides a mathematical tool to define and comprehensively describe all metabolic routes that are both stoichiometrically and thermodynamically feasible for a group of enzymes. The optimal metabolic flux distribution and the corresponding pathways are identified by LP optimisation subjecto to the stoichiometric flux balance analysis (FBA) and the constrains on negative gibbs free energy change, for achieving the maximum yield of products. In the second level, thermodynamic optimisation in terms of the Gibbs free energy change minimisation is carried out for the best performance of the system. The Gibbs free energy changes are predicted for the stoichiometrically balanced sequences of pathways from for the

energetic coupling between metabolites. The Gibbs free energy of metabolites in this model is presented as a function of temperature, pressure, pH and metal ions concentrations. Hence the minimisation of the Gibbs free energy change optimises these operating conditions, for the optimal pathways that achieve the desired objectives on productions. This two stage optimisation approach is integrated through the flux balance analysis, the elementary flux mode analysis, the inequality constraints on the negative Gibbs free energy change and the calculation of metabolites formation gibbs energy. The optimisation procedure thus generated ensures the maximisation of the external flux capacities and the minimisation of the Gibbs free energy change and hence derives the optimal pathways and the operating conditions for achieving the desired objectives. The methodology is demonstrated by a case study on the optimisation of pentose phosphate pathway (PPP) and the glycolysis cycle of the insilico Escherichia coli. The yield of amino acid is maximised, while the minimisation of the Gibbs free energy change during the process optimises the operating conditions required for the process. Thus the optimal flux distributions as well as the optimal conditions on the pH and ion concentrations are achieved.

Keywords: metabolism optimisation, Gibbs free energy minimisation, thermodynamic analysis, pathway analysis, FBA

Introduction

A metabolic network is most often interpreted and modeled in terms of a collection of enzyme-catalyzed reactions with transport phenomena that utilise substrate metabolites to generate final metabolites. Metabolic flux analysis (MFA) is most widely adopted for rational design and in silico engineering of metabolic system to calculate the nonmeasurable quantities from measurable quantities using a stoichiometric model with a given set of metabolic reactions and mass balances around the metabolites. Therefore, to compute the flux distribution in a metabolic system, flux balance analysis (FBA), based on linear programming (LP), has been firmly established theoretically (Varma and Palsson, 1994a). In general, FBA provides one desired physiological endpoint, e.g., the maximum growth rate, and its corresponding flux distribution under some culture conditions. The unknown fluxes within a metabolic reaction network are evaluated by LP, subject to constraints pertaining to mass conservation, thermodynamic properties, and capacity as described elsewhere (Edwards et al., 1999; Varma and Palsson, 1994a). The application of flux balance analysis has been effectively dealt with metabolic networks of various kinds (Edwards et al., 1999; Schilling et al., 1999)

However, several critical issues remain unresolved, especially the uniqueness of flux distribution. The implementation of LP in FBA frequently leads to multiple (or alternate) optima depending on the initial starting point, thereby signifying the existence of multiple solutions. Hence, the complexity of the metabolic reaction systems necessitates the development of the integrated approaches to analyse and interpret the systemic properties of cellular metabolism, which shifts the emphasis from single metabolic reactions to systemic pathways. The theoretical foundation for identification of a unique set of

systemically independent biochemical pathways, termed extreme pathways, lies on the stochiometry and thermodynamic limitations of systems. All these pathways should be regarded as the true functional units of metabolic systems, which can also be used to represent any flux distribution achievable from flux balance analysis. These works propose a unified approach for identifying multiple flux distribution in metabolic flux analysis, through the combination of flux balance model based on LP and pathway analysis based on elementary flux modes analysis. Therefore, the application of pathway analysis, together with the FBA, plays a critical role in the improvement of metabolic flux analysis.

Thermodynamic analysis can be used to decide whether a given microbial growth or metabolic reaction is feasible and find the optimal operating conditions for achieving the maximum productivity from bioprocesses. Based on such analysis, it ought to be possible to estimate the key parameters in biotechnological cultures and thus to address the operating viability of bioprocesses. Once the first measurement is carried out, thermodynamic analysis based predictions can be used as benchmarks, indicating the scope for improvement. All of these would be of invaluable help in bioprocess development (von Stockar, 2005).

In thermodynamic terms, the difference in Gibbs free energy sets the driving force for any system undergoing changes. The Gibbs free energy change needs to be negative for any phenomena to be feasible. When a system is in equilibrium the Gibbs free energy change of the system is the minimum. Thus, any system will change towards a minimum Gibbs free energy change. In broad terms, thermodynamic analysis of a process involves the determination of the Gibbs free energy change, the enthalpy change and the entropy

change under specified conditions. The use of Gibbs free energy is appropriate under conditions if process occurs spontaneously (i.e. irreversibly). In this case the Gibbs free energy change is negative. This is the basis for assessing the thermodynamic feasibility of a process. There is also a universal law that a reaction in which the free energy change is large and negative has an equilibrium that favours the side of products. Thus, thermodynamic analysis based on the driving force for microbial growth in terms of Gibbs energy change can be put in practical use in evaluating the performance of microorganisms with respect to growth and bioproduct synthesis.

In this work, a novel methodology is developed to establish an integrated metabolic engineering approach for the elucidation and optimisation of metabolic systems. Not only the performances of metabolic systems in terms of productivity are optimised by combined flux balance analysis and pathway analysis, but also the optimal operability is achieved by the introduction of thermodynamic otpimisation approach. To achieve this, a bi-level optimisation strategy is developed. Application of thermodynamic analysis based on Gibbs free energy change in optimisation approach is novel and based on heuristic, which turns thermodynamics into a useful tool in metabolic engineering. The methodology has been detailed in the first part of this paper. This includes the formulations and illustrations on Gibbs free energy change, the metabolic flux analysis and the algorithm for bi-level optimisation. In the second part, a case study on the metabolism network of pentose phosphate pathways (PPP) and glycolysis cycle of in silico Escherichia coli has been established to demonstrate the profound efficacy of this methodology.

Problem statement

For a given metabolic system, the objective of this work is to evaluate and optimise its performance and metabolic flux distribution in terms of the yields of desired products and the rational operating conditions that facilitate achieving the maximum yields of desired products. We propose an optimisation based methodology that includes the maximum productivity of a metabolic network, the optimal flux distribution attaining the maximum productivity, and the optimal operating conditions for the system (Fig. 1). The results achieved should be thermodynamically and stoichiometrically feasible for metabolic systems, and practically realisable and rational for industrial applications. The methodology has been illustrated by the demonstration of a practical example.

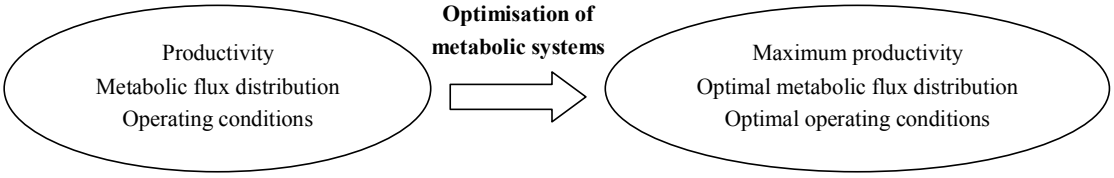

Fig. 1 Problem statement

Methods

1. Gibbs free energy change for biochemical reaction system

There are two kinds of reaction equations, chemical equations which are written in terms of species and balance elements and charge, and biochemical equations written in terms of biochemical reactants at a specified pH that are bounded by species in equilibrium.

However, biochemical equations do not balance elements that are assumed fixed, such as hydrogen at a constant pH. The conventional thermodynamic analysis thus can not provide the criteria for spontaneity or equilibrium. Therefore, it is necessary to define new transformed thermodynamic properties for biochemical equations (Alberty, 1994). These new thermodynamic properties of biochemical reactants, especially Gibbs free energy changes, can be calculated directly from the standard thermodynamic properties of their corresponding species as a function of pH and ionic strength *I* (Alberty, 1994).

2. Metabolic system analysis

The metabolic system model can be described by flux balance analysis. The tool of pathway analysis is used for identifying all the elementary path modes included in a system. A combination between them is introduced for the development of integrative methods to analyse and interpret metabolism networks (Fig. 2).

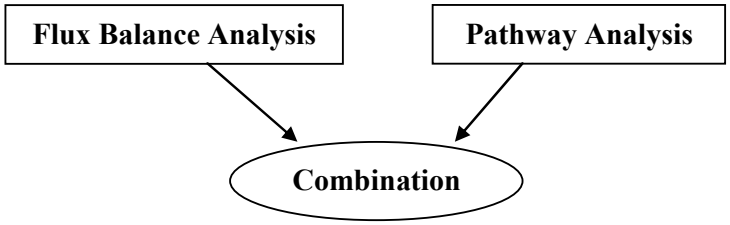

Fig. 2 Metabolic system analysis

2.1 Flux balance analysis

A metabolic network is a collection of enzyme-catalysed reactions and transport processes that serve to dissipate substrate metabolites and generate final metabolites (Schilling, 2000). Flux balance analysis can be used to describe metabolic system models that include a complete list of reactions as well as metabolites and cofactors, in a

quantitative manner. Metabolites are classified as internal or external according to whether or not they are to fulfill the pseudo-steady state condition. In other words, the total production rate of each internal metabolite equals to its consumption rate, while external metabolites (which are alternatively called pool metabolites, or sources and sinks) do not satisfy this condition. The number of mass balance is the same as the number of internal metabolites. For the flux balance analysis of metabolic systems, the only information required is the stoichiometry of metabolic reactions, the mass balance around the internal metabolites under pseudo-steady state and the external metabolite sources uptake. The process of flux balance analysis is illustrated in Fig. 3.

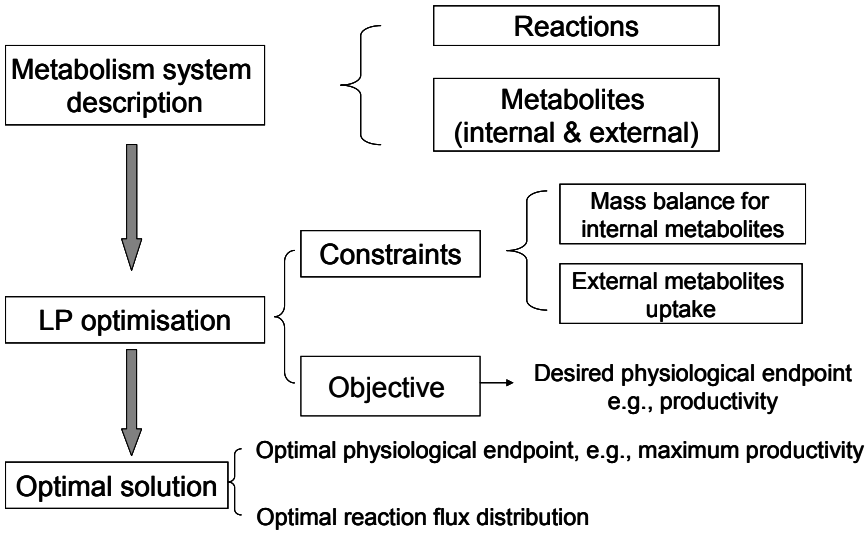

Fig. 3 Flux balance analysis

2.2 Pathway analysis

A unique set of systemically independent biochemical pathways, termed extreme pathways, based on the stochiometric and thermodynamic feasibilities of metabolic systems for a group of enzymes is identified. As a true functional unit of metabolic

systems, each pathway represents a full set of nondecomposable steady-state flows that a network can support. The enumeration of all feasible pathways within a metabolic system can be accomplished using the elementary flux mode (EFM) analysis algorithm (Schuster 2000), implemented in MATLAB Version 6.5 (Mathworks Inc., Natick, MA).

2.3 Combination between pathway analysis and flux balance analysis

The complexity in metabolic systems necessitates the development of the integrated approaches to analyse and interpret the systemic properties of cellular metabolism, which emphasises not only on single metabolic reactions, but also the systemic pathways defined by the elementary flux mode analysis. Therefore, we derived a combination of pathway analysis and metabolic flux analysis based on the relationship between reaction flux distribution $\overline{V}$ and pathway flux distribution $\overline{B}$.

3. Bi-level optimisation

A bi-level optimisation approach is developed, which combines the metabolic flux analysis and pathway identification with the thermodynamic analysis of metabolic systems (Fig. 4). In Module 1, the objective of productivity of external metabolites is maximised by linear programming, which is subjected to mass balance equations derived from pathway and flux analysis and the negative Gibbs free energy change inequalities for feasibility of pathways. In Module 2, thermodynamic optimisation in terms of the minimisation of the total Gibbs free energy change of a metabolic system is carried out to predict the optimal operating conditions for the system. The objective function of thermodynamic optimisation is expressed by means of pathway Gibbs free energy change

as well as the optimal pathway flux distribution derived from Module 1.

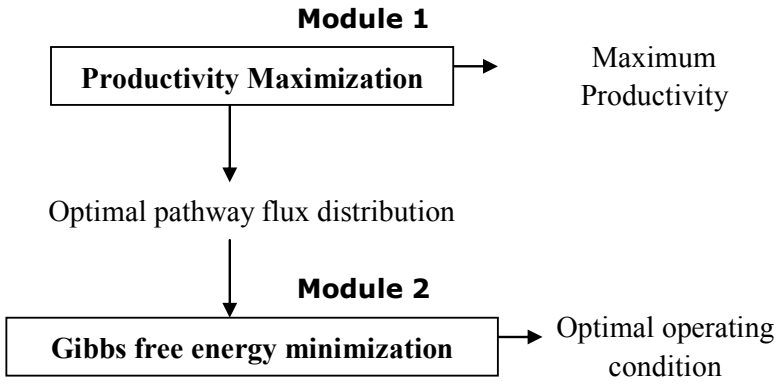

Fig. 4 Modules of Bi-level Optimisation Approach

Case Study

A case study on the synthesis of pentose phosphate pathways (PPP) and glycolysis cycle of *in silico* model of *Escherichia coli* metabolism has been constructed to illustrate the proposed bi-level optimisation approach.

1. The representation of the metabolic network

The metabolism network under consideration is embedded with the glycolytic pathway and the pentose phosphate pathway in the *in silico* model of *E. coli* metabolism. This network incorporates 26 metabolites (4 external metabolites, 15 internal metabolites, 7 cofactors) and 19 metabolic reactions. (Tables 1-2). The overview of the reaction scheme for the model is indicated in Fig.5.

Table 1. Metabolic reactions of glycolysis circle and PPP circle in *E.coli* model

Enzyme	Gene	Rxn no.	Reaction
Glycolysis(10)			
Phosphoglucose isomerase	pgi	1	$G6P \leftrightarrow F6P$
Phosphafructokinase	pfkA	2	$F6P + ATP \rightarrow ADP + FDP$
Fructose-1,6-bisphosphatase	fbp	3	$FDP \rightarrow F6P + PI$
Fructose-1,6-bisphophate aldolase	fba	4	$FDP \leftrightarrow T3P1 + T3P2$
Triosphosphate isomerase	tpiA	5	$T3P1 \leftrightarrow T3P2$
Glyceraldehyde-3-phosphate dehydrogenase	gapA	6	$T3P1 + PI + NAD \rightarrow NADH + 13PDG$
Phosphoglycerate kinase	pgk	7	$13PDG + ADP \rightarrow ATP + 3PG$
Phosphoglycerate mutes	gpmA	8	$3PG \leftrightarrow 2PG$
Enolase	eno	9	$2PG \leftrightarrow PEP$
Pyruvate kinase	pyk	10	$PEP + ADP \rightarrow ATP + PYR$
Pentose phosphate pathway (PPP)(9)			
Glucose-6-phosphate dehydrogenase	zwf	11	$G6P + NADP \rightarrow NADPH + D6PGL$
6-Phophogluconolactonse	pgl	12	$D6PGL \leftrightarrow D6PGC$
6-Phosphogluconate dehydrogenase	gnd	13	$D6PGC + NADP \rightarrow NADPH + CO_2 + RL5P$
Ribose-5-phosphate isomerase	rpiA	14	$RL5P \leftrightarrow R5P$
Ribulose phosphate 3-epimerase	rpe	15	$RL5P \leftrightarrow X5P$
Transketolase 1	tktI	16	$X5P + R5P \leftrightarrow T3P1 + S7P$
Transaldolase	tal	17	$T3P1 + S7P \leftrightarrow E4P + F6P$
Transketolase 2	TktII	18	$X5P + E4P \leftrightarrow F6P + T3P1$
5-Phosphoribosyl-1-pyrophosphate synthetase	Prs	19	$R5P \rightarrow R5Pex$

Among the external metabolites, glucose-6-phosphate is considered as the source because it is formed from glucose taken up into the cell. This is regarded as the only carbon source consumed through the system while producing metabolic products. Other external metabolites include carbon dioxide, Ribose *5*-phosphate, and pyruvate, while pyruvate is assumed as the objective sink for the productivity maximisation.

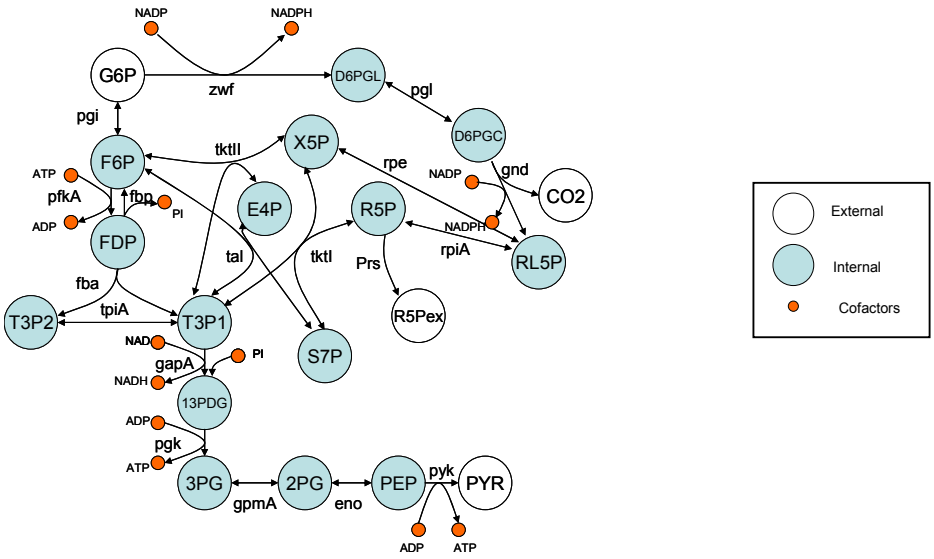

Fig. 5 Overview of the metabolic network of Glycolysis Circle and PPP circle in *E.coli* model

Table 2. Metabolites of glycolysis circle and PPP circle in *E.coli* model

Abbreviation	Compound
External metabolites(4)	
G6P	Glucose *6*-phosphate
PYR	Pyruvate
CO_2	Carbon dioxide
R5Pex	Ribose *5*-phosphate (external)
Internal metabolites(15)	
F6P	Fructose *6*-phosphate
FDP	Fructose *1, 6*-diphosphate
*T3P*1	Glyceraldehyde-*3*-phosphate
*T3P*2	Dihydroxyacetone phosphate
13*PDG*	*1, 3-P-d* glycerate
3*PG*	*3-P-d* glycerate
2*PG*	*2-P-d* glycerate
PEP	Phosphoenolpyruvate
D6PGL	*d-6*-Phosphogluconate
D6PGC	*d-6*-Phosphoglucono-δ-lactone
RL5P	*d*-Ribulose *5*-phosphate
R5P	Ribose *5*-phosphate
X5P	Xylulose-*5*-phosphate
S7P	*d*-Sedoheptulose-*7-P*
E4P	Erythrose *4*-phosphate
Cofactors(7)	
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
NAD *NADH*	Nicotinamide adenine dinucleotide
NADP *NADPH*	Nicotinamide adenine dinucleotide phosphate
PI	Phosphate

1.1. Pathway and metabolic analysis of the metabolism network

Formulation of the pathway analysis in a network has been described previously (Scheuster, 2000). In this research, a set of metabolic pathway modes has been constructed by using elementary mode analysis, in order to systematically organise and analyse the metabolic network. Thirteen elementary path modes are derived from computation (Table 3). Fig.6 describes the pathway mode 3 for example to illustrate the reactions involved in this pathway.

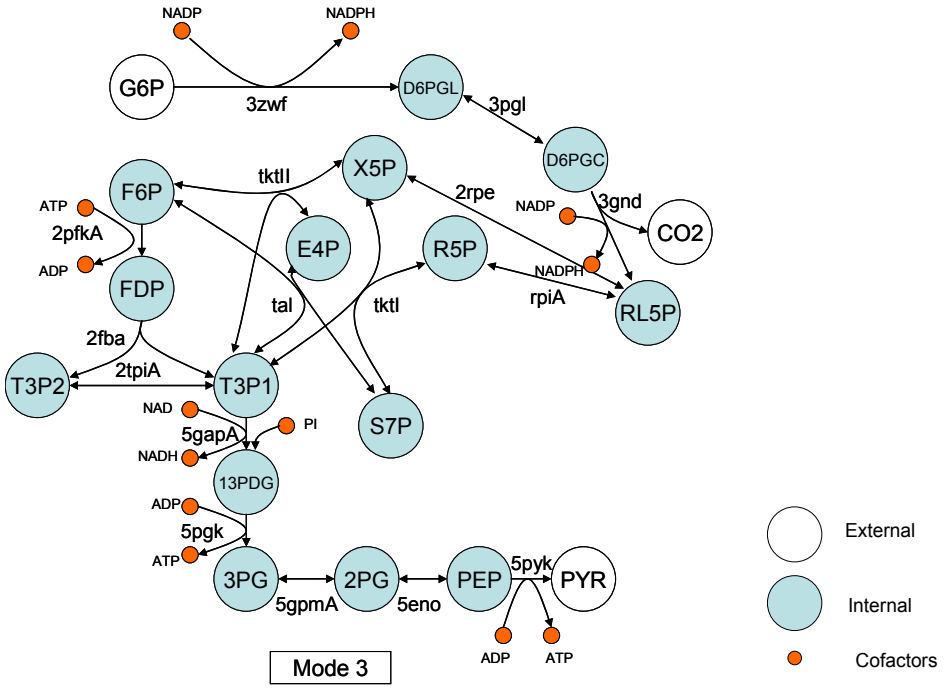

Fig. 6 Graphical representation of the pathway mode 3 pertaining to the reaction scheme

Table 3. Elementary path modes of the combined glycolysis and PPP system

Mode	Overall equation	Steps
1	$G6P+3ADP+2Pi+2NAD \rightarrow 3ATP+2NADH+2Pyr$	pgi, pfkA, fba, tpiA, 2gapA, 2pgk, 2gpmA, 2eno, 2pyk
2	$G6P+2ADP+Pi+NAD+6NADP \rightarrow 2ATP+NADH+6NADHP+3CO_2+Pyr$	-2pgi, gapA, 3zwf, 3pgl, 3gnd, rpiA, 2rpe, tktI, tal, tktII, pgk, gpmA, eno, pyk,
3	$3G6P+8ADP+5Pi+5NAD+6NADP \rightarrow 8ATP+5NADH+6NADHP+3CO_2+5Pyr$	2pfkA, 2fba, 2tpiA, 5gapA, 3zwf, 3pgl, 3gnd, rpiA, 2rpe, tktI, tal, tktII, 5pgk, 5gpmA, 5eno, pyk
4	$G6P+2NADP \rightarrow 2NADPH+CO_2+R5Pex$	zwf, pgl, gnd, rpiA, Prs
5	$5G6P+ATP \rightarrow ADP+6R5Pex$	5pgi, pfkA, fba, tpiA, 4rpiA, -4rpe, -2tktI, -2tal, -2tktII, 6Prs
6	$G6P+12NADP \rightarrow 12NADPH+Pi+6CO_2$	-5pgi, -fba, -tpiA, 6zwf, 6pgl, 6gnd, 2rpiA, 4rpe, 2tktI, 2tal, 2tktII, fbp
7	$ATP \rightarrow ADP+Pi$	pfk, fbp
8	$2ADP+Pi+NAD+3R5Pex \rightarrow 2ATP+NADH+2G6P+Pyr$	-2pgi, gapA, -2rpiA, 2rpe, tktI, tal, tktII, pgk, gpmA, eno, pyk, -3Prs
9	$2ADP+Pi+NAD+4NADP+R5Pex \rightarrow 2CO_2+2ATP+NADH+4NADHP+Pyr$	-2pgi, gapA, 2zwf, 2pgl, 2gnd, 2rpe, tktI, tal, tktII, pgk, gpmA, eno, pyk, -Prs
10	$8ADP+5Pi+5NAD+3R5Pex \rightarrow 8ATP+5NADH+5Pyr$	2pfkA, 2fba, 2tpiA, 5gapA, -2rpiA, 2rpe, tktI, tal, tktII, 5pgk, 5gpmA, 5eno, 5pyk, -3Prs
11	$8ADP+5Pi+5NAD+4NADP+2G6P+R5Pex \rightarrow 2CO_2+8ATP+5NADH+4NADHP+5Pyr$	2pfkA, 2fba, 2tpiA, 5gapA, 2zwf, 2pgl, 2gnd, 2rpe, tktI, tal, tktII, 5pgk, 5gpmA, 5eno, 5pyk, -Prs
12	$6R5Pex \rightarrow 5G6P+Pi$	-5pgi, fbp, -fba, -tpiA, -4rpiA, 4rpe, 2tktI, 2tal, 2tktII,-6 Prs
13	$8NADP+2R5Pex \rightarrow 4CO_2+8NADPH+G6P+Pi$	-5pgi, fbp, -fba, -tpiA, 4zwf, 4pgl, 4gnd, 4rpe, 2tktI, 2tal, 2tktII, -Prs

Based on the results of pathway analysis, a 19×13 stoichiometric matrix $\overline{A}$ is derived from the stoichiometry of reactions in each pathway and a 13×11 stoichiometric matrix $\overline{U}$ is derived from the stoichiometry of external metabolites and cofactors in each pathway.

1.2 Thermodynamic properties of the external metabolites and cofactors

All the biochemical species of the external metabolites and cofactors involved in the network (Fig. 5) as well as their corresponding thermodynamic properties of the standard formation Gibbs free energy at 25 ℃, 1 bar and I=0, are illustrated in Table 4.

Table 4. Standard formation Gibbs free energy, charges and hydrogen atom numbers for species

Species	$\Delta G_f^{\,o} / kJ \cdot mol^{-1}$	charge z_i	hydrogen atom numbers $N_i(H)$
$G6P^{2-}$	-1763.94	2	11
PYR^{-}	-472.27	1	3
$R5P^{2-}$	-1605.34	2	9
NAD^{-}	0	1	26
$NADH^{2-}$	22.65	2	26
$NADP^{3-}$	0	3	25
$NADPH^{4-}$	25.99	4	25
HPO_4^{2-}	-1095.1	2	1
$H_2PO_4^{-}$	-1137.3	1	2
ATP^{4-}	−2573. 49	4	12
$HATP^{3-}$	−2616. 87	3	13
H_2ATP^{2-}	−2643. 58	2	14
ADP^{3-}	−1711. 55	3	12
$HADP^{2-}$	−1752. 53	2	13
H_2ADP^{-}	−1777. 42	1	14

Firstly, the standard formation Gibbs free energy of these species, at a specific pH and ionic strength ($pH = 5$, $I = 0.2$) is calculated from Eq.3 and 8 respectively, based on their hydrogen atom numbers $N_i(H^+)$ and the charge z_i (Table 4). Next, the standard formation Gibbs free energy for all the external metabolites and cofactors within the system, like ATP, ADP and inorganic phosphate, are calculated and shown in Table 5.

Table 5 Standard formation Gibbs free energy for external metabolites and cofactors

External metabolites and cofactors	$\Delta G_f'^{o} / kJ \cdot mol^{-1}$
G6P	-1442.52
PYR	-385.163
R5Pex	-1342.5
NAD	760.7564
NADH	783.4064
NADP	731.4673
NADPH	757.4573
PI	-540.628
ATP	-1087.29
ADP	-711.984

2. Productivity maximisation

For the productivity maximisation, the objective function is to maximise the product flux of pyruvate using the flux balance analysis (FBA). The uptake rate of glucose-6-phosphateis is specified to be 10 $mmol/gDCWh$. The optimal flux distribution of pathways is shown in Table 6. The total Gibbs free energy change for this process at this operating conditions ($pH = 5$, $I = 0.2$) is -1321 $kJ \cdot mol^{-1}$.

Table 6 Optimal flux distribution for pathways

Mode	Metabolic Flux B(p)
1	0
2	10
3	0
4	0
5	0
6	0
7	0
8	0
9	0
10	0
11	0
12	0
13	0

3. Gibbs free energy minimisation

To predict the optimal conditions for operation, the optimal flux distribution achieved by the productivity maximisation is used as the input for thermodynamic evaluation. The standard formation Gibbs free energy of the external metabolites and cofactors within the system as functions of pH, ionic strength *I* and the standard formation Gibbs free energy of their corresponding species are predicted. The expression of the standard pathway Gibbs free energy change for the 13 pathway modes are derived from the stoichiometry

of the overall reaction equations of pathways. The NLP optimisation program is run under the GAMS modeling environment. The objective function is the minimisation of the total Gibbs free energy for all the pathways included. The result of the Gibbs free energy minimisation is presented in Table 7. The optimal operating conditions obtained are $pH = 7$ and $I = 0.3$. The minimum Gibbs free energy change for this metabolic network is -5577.197 $kJ \cdot mol^{-1}$, which is significantly minimised compared to the initial value (-1321 $kJ \cdot mol^{-1}$).

Table 7. Standard formation Gibbs free energy at optimal condition

External metabolites and cofactors	$\Delta G_f'^{opt} / kJ \cdot mol^{-1}$
G6P	-1318.634
PYR	-350.745
R5Pex	-1241.617
NAD	1059.736
NADH	1079.833
NADP	1012.137
NADPH	1032.171
PI	-1228.872
ATP	−2361. 616
ADP	−1493. 225
$\Delta G_{tot}^{opt} / kJ \cdot mol^{-1}$	−5577. 197

Conclusion

A novel bi-level optimisation methodology has been presented for the productivity and thermodynamic performance of metabolic systems. The theoretical connection between flux balance analysis and pathway analysis is well established. Their combined application has been integrated with the thermodynamic constrains for metabolic flux analysis in order to predict the maximum productivity of the desired products. The corresponding optimal metabolic flux distribution is achieved. Moreover, thermodynamic

optimisation in terms of the Gibbs free minimisation has been successfully developed for metabolic systems, from which, the best operation conditions for the optimal flux distribution are predicted. This work proposed a rational approach for predicting and optimising the performance of metabolic systems. The heuristic idea of introducing thermodynamic analysis into metabolic engineering presents a new way to rationalise metabolic pathway analysis, hence, providing a better control mechanisms for metabolic systems and to find the best operating conditions for industrial bioprocesses.

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