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Study of Metabolic Flux Distribution in Rice (*Oryza sativa*) Cultures for Starch Production

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ABSTRACT

The demand for starch-rich crops remains high due to their wide applications, and one of them is rice (*Oryza sativa*). However, large-scale rice production faces challenges such as unstable productivity, climate changes and excessive use of agrochemicals. Plant cell culture technology is proposed to increase rice yield and produce a drought-resistance variety of rice to sustain its demand. However, the amount of starch in rice cultures is expected to be smaller compared to the planted ones. The main aim of this study is to apply Flux Balance Analysis (FBA) to optimize starch production in rice cultures. This study reconstructed the stoichiometric metabolic model for rice culture based on the published articles. It consists of 160 reactions and 148 metabolites representing rice's main carbon metabolism towards starch production. The model was then formulated in GAMS v31.1.0, and the objective function was set to the maximization of biomass and starch. The selected constraints (sugar uptake rates and cell growth rates) from previous studies were utilized. The simulated starch production rate values were achieved at the highest glucose uptake rates with the value of 0.0544 mol/g CDW.h. The internal metabolic flux distributions demonstrated that the incoming carbon fixes were directed towards the glycolysis pathway, TCA cycle, PPP cycle, and starch biosynthesis reactions. The study results serve as a starting point to further understanding the starch production mechanism in plants known to be complex.

Keywords: FBA, General Algebraic Modeling System (GAMS) software, metabolic flux distribution, rice, starch

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INTRODUCTION

People have utilized starchy materials derived from seeds, roots, and tubers in the past and even today for various purposes. Rice (*Oryza sativa*), the commonly cultivated rice species, is one of the prominent sources of starch used as a source of human food, feed for livestock, and raw materials for chemical, food, and pharmaceutical industries. The most abundant component in rice is carbohydrates consisting of approximately 80% of starch (Marco et al., 2008). Starch is a natural biopolymer consisting of amylose and amylopectin, the polymer type of glucose. The proportions of amylose and amylopectin in rice differ depending on the rice's variety, and these proportions determine the physical properties and functionality of the rice. It has been approximated that the amount of amylose and amylopectin in the rice starch is 15–20% and 80–85%, respectively (Shu et al., 2007).

With the growing demand for rice, many initiatives focus on plant tissue culture to stabilize the demand with the infeasibility of large-scale production, which require several thousand hectares of arable land. Plant tissue culture is known for its ability to produce plants with a higher yield and its flexibility to cultivate the plant *in vitro,* which contributes to overcoming the biotic and abiotic factors affecting the growth of intact plants in the environment. Therefore, many cultures have been used to produce many plants, including rice itself. However, it is expected that the starch content in the plant culture of rice is lesser than the real plant. It is because plant tissue culture involves the growth of a cell, not a whole plant, and certain enzymes or genes may not exist to support starch production.

Flux Balance Analysis (FBA) is a method of metabolic modeling that utilizes a constraint-based approach. The prediction of flux distribution in a metabolic network can be achieved by using this method through maximization and minimization of an objective function through the utilization of experimental data to validate the model predictions (Orth et al., 2010). Through FBA, knowledge about the system's metabolic concentrations and enzyme kinetics is not required as it merely relies on stoichiometric characteristics. Additionally, the assumption of the steady-state system in FBA removes the need to determine rate laws and kinetic parameters, which are commonly difficult to identify (Raman & Chandra, 2009). However, reports on FBA on starch production are still scarce as most studies on FBA focus on optimizing biomass production. Therefore, this study aimed to utilize FBA to optimize starch production in rice plant cell cultures by observing its flux distributions in the constructed metabolic pathway network using GAMS 33.1.0 software.

METHODS AND MATERIALS

System Definition

Metabolic networks for rice (*Oryza sativa*) were reconstructed in matrix form by modifying the primary metabolism pathways in Puad (2011), focusing only on the starch metabolism.

The modification was done based on literature reading (Shaw & Kundu, 2015; Zeeman et al., 2010).

Development of Mass Balance for Each Metabolite

After identifying all metabolites involved in the starch synthesis, mass balances containing all reactions were developed. Mass balances were described using a stoichiometric matrix, S, and the flux matrix, v. The stoichiometric representation for each reaction in the form of a numerical matrix, is as shown in Equation 1. Metabolites produced have a positive sign, and the metabolites consumed will have a negative sign.

$$S \,.\, v = 0 \qquad (1)$$

The assumption in this model was steady-state metabolic flux to ensure the metabolic concentrations remain constant; thus, each metabolite has one linear equation equal to zero (Orth et al., 2010).

Defining Constraints

Typically, in FBA, there will be more reactions or specific unknown fluxes than the metabolites. Thus, the steady-state solution for the metabolic fluxes is underdetermined. Furthermore, it leads to complexity in the metabolic network, increasing the number of unknown fluxes. Thus, additional constraints are required to ensure a realistic flux distribution can be predicted in FBA (Lularevic et al., 2019). In this study, the experimental constraint was sugar uptake rate by rice cell cultures, while biomass composition was used for biological constraint.

Biomass composition is described in terms of macromolecular components of cells, and the composition is varied for different species, which mainly depends on the growth conditions and environment (Carnicer et al., 2009; Hanegraaf & Muller, 2001). The macromolecular composition of cells can be divided into seven components which are: (1) RNA; (2) DNA; (3) proteins; (4) phospholipids; (5) fatty acids; (6) carbohydrates; (7) starch. Although starch is a polymeric carbohydrate, it is categorized as a storage polysaccharide in the reconstructed metabolic model. At the same time, primary cell wall components (cellulose, hemicellulose, and pectin) contribute to the carbohydrate percentage of 1 g of the cell (Table 1). Due to limited information on 1 g of rice macromolecular cell composition, this model used the macromolecular composition of plant cells collated from different plant types, as summarized in Table 1.

The experimental data for sugar uptake rates were obtained from the initiation of rice cell suspension culture reported in the previous study (Lakshmanan et al., 2013). The rice suspension cultures were established from calli induced by rice seed on an amino acid callus induction medium containing 2,4-dichlorophenoxyacetic acid (2 mg/L), sucrose (30 g/L),

Table 1
Molecular composition of different plant cells for applications in this model

Macromolecules	gg^{-1} cell DW	% of g cell DW	Plant cells	References
RNA	0.03	3	*Arabidopsis thaliana*	(Poolman et al., 2009)
DNA	0.03	3	*Arabidopsis thaliana*	(Poolman et al., 2009)
Proteins	0.042	4.2	Rice	(Amthor, 2000)
Phospholipid	0.027	2.7	*Catharanthus roseus*	(Toivonen et al., 1992)
Fatty acids	0.014	1.4	Rice	(Amthor, 2000)
Carbohydrates	0.55	52	Rice	(Amthor, 2000)
Starch	0.307	33.7	Rice	(Amthor, 2000)

and Gelrite (2 g/L) for two months. The cultures were incubated at 28°C and 120 rpm in a gyratory shaking incubator. Experiments were carried out in 100-mL Erlenmeyer flasks with 30 mL of medium (Lakshmanan et al., 2013). These experimental data are required as the constraints to determine the intracellular flux distribution during the optimization of starch production in rice (Table 2). The data were taken at the stationary phase, from 144 h to 336 h due to sugar uptake rates at these points. In relation to that, these points are required to be fed separately into the model to observe if there is any significant impact on the objective function upon the change in the specific growth rate of rice cultures at different times of the growth curve.

Table 2
Calculated experimental specific rates for cell suspension culture of rice from a previous study (Lakshmanan et al., 2013) that were used as the constraints for FBA in this study

Time (h)	Specific growth rate, $\mu_{experimental}$ (h^{-1})	Specific sucrose uptake rate, q_S (mmole sugar per gDW.h)	Specific glucose uptake rate, q_G (mmole sugar per gDW.h)	Specific fructose uptake rate, q_F (mmole sugar per gDW.h)
48	0.00556	0.0325	0	0
96	0.0114	0.2326	0	0
144	0.0052	0.0609	0.0289	0.0289
192	0.0022	0	0.0122	0
240	0.0038	0	0.0105	0.0210
288	0	0	0.0193	0.0289
336	0	0	0	0.0101

Optimizing Steps and Simulations in GAMS

Numerous objective functions can be set in GAMS; specifically, in this study, they maximized biomass and starch production rates. By clicking the 'Run' button in GAMS, it executes an output file that lists the fluxes' value upon optimizing the objective function. The values are then presented in a metabolic flux map drawn manually in Microsoft Excel 2019.

RESULTS AND DISCUSSION

Reconstruction of Plant Primary Metabolic Pathways for Starch Production

For the development of the stoichiometric metabolic model in this study, plant primary metabolic pathways described by Puad (2011) have been used and reconstructed based on the information collected from various sources such as published articles, books, and websites. Upon reconstruction, the developed stoichiometric model for rice consists of 160 reactions and 148 metabolites. From the stoichiometric models, all mass balances developed for each metabolite were formulated in GAMS v33.1.0. The alteration of the plant's primary pathways involved only the addition of the starch metabolism reactions, which is the main objective of this study. The model is composed of central metabolism networks such as glycolysis, Pentose Phosphate Pathway (PPP), and TCA cycle, where these metabolisms take part mainly in the synthesis of biomass such as fatty acids, starch, protein, carbohydrates, amino acid, and phospholipids (Table 3).

Table 3
Number of reactions and metabolic pathways involved in this study

Name of Pathway	Number of Reactions	Name of Pathway	Number of Reactions
Protein Synthesis	58	RNA Synthesis	1
DNA Synthesis	1	Carbohydrate Synthesis	13
Transport Reactions	13	Central Carbon Metabolism	49
Phospholipid biosynthesis	14	Transhydrogenase reactions	2
Biomass constituent	1	ATP consumption for maintenance	1
Fatty acid biosynthesis	6	Carbamoyl phosphate formation	1

Optimization of Starch Production in Rice Utilizing FBA with Maximization of Biomass and Starch Production Rate as the Objective Function

As discussed previously, this study utilized the experimental data on the initiation of rice cell suspension (Lakshmanan et al., 2013) to simulate the starch production mechanism compared to other types of tissue culture due to the condition for the initiation of the culture itself. A liquid culture system such as cell suspension culture provides a better approach for plant tissue culture processes such as micropropagation and regeneration. Continuous shaking during incubation serves good aeration, oxygen, and nutrient availability. Furthermore, it demonstrates that cells in suspension culture have better contact and a large surface area of the cells directly exposed to the supplied liquid medium. Sucrose uptake rates, glucose uptake rates and fructose uptake rates from Lakshmanan et al. (2013) were fed into the model as the experimental constraints to simulate the starch production mechanism. Since there is a lack of data for other components such as oxygen consumption,

nitrate uptake rates and sulfate uptake rates, they were left as free variables, which GAMS itself determine.

Based on the simulation results, with different objective functions being implemented, the flux distributions were still the same in both cases. Starch is expected to be part of biomass composition (Table 1). The highest values of biomass (0.0135 mmol/g CDW.h) and starch production (0.0041 mmol/g CDW.h) were obtained at 144 h (Day 6) of growth which is during the exponential phase. During this growth period, sugar uptake rates, specifically glucose uptake rates, are at the highest value, which explains the highest value of starch production attained on that day. It is due to the reason that the starch structure is made of a chain of glucosyl compounds as starch is produced from sugars. During photosynthesis, plants will convert light energy into either chemical energy or sugar; this sugar will then either be stored in the form of starch or utilized for respiration and growth. It is reflected in a report where the starch content in callus culture is correlated with the glucose content (Lee & Huang, 2013).

Figure 1 shows the distribution of fluxes in rice cultures for starch and biomass production optimization, where the yellow-colored value shows that no flux was present and the green colored shows the presence of flux. The incoming carbon fluxes from glucose and fructose were directed from glycolysis to the TCA cycle, PPP, carbohydrates biosynthesis, and starch biosynthesis. Based on the flux distributions in Figure 1, fructose 6-phosphate (F6P) is converted to glucose 6-phosphate (G6P) and then glucose 1-phosphate G1P before ADPglucose, which is the precursor to initiating starch biosynthesis, which agrees with Pfister & Zeeman (2016).

Shaw and Kundu (2015) have developed a model for rice leaf metabolism where they emphasized the flux distribution for high and low starch production in the central carbon metabolism. In their study, the maximum and minimum value of starch was obtained using biomass optimization criteria, while in this study, it is for the maximization of starch production. Compared to what was developed in this study, the starch produced is still low compared to the value of starch produced in Shaw and Kundu (2015). It is expected because this study involved the distribution of fluxes in plant cell culture, which is not the whole plant.

Additionally, through the comparison of flux distribution for minimum and maximum starch production, Shaw and Kundu (2015) also mentioned that in the case of maximum starch production, a major fraction of glyceraldehyde 3-phosphate (GL3P) is converted to G6P to overproduce starch, and in the case of minimum starch production, GL3P is converted mostly to 3-phosphoglyceraldehyde (PG3) instead of G6P. G6P is a precursor for G1P production, which is the precursor for ADPGlucose production. In contrast with this study, the reaction for the conversion of GL3P to PG3 is active, and there is a lack of reaction for the conversion of GL3P to G6P. Furthermore, our model did not include the

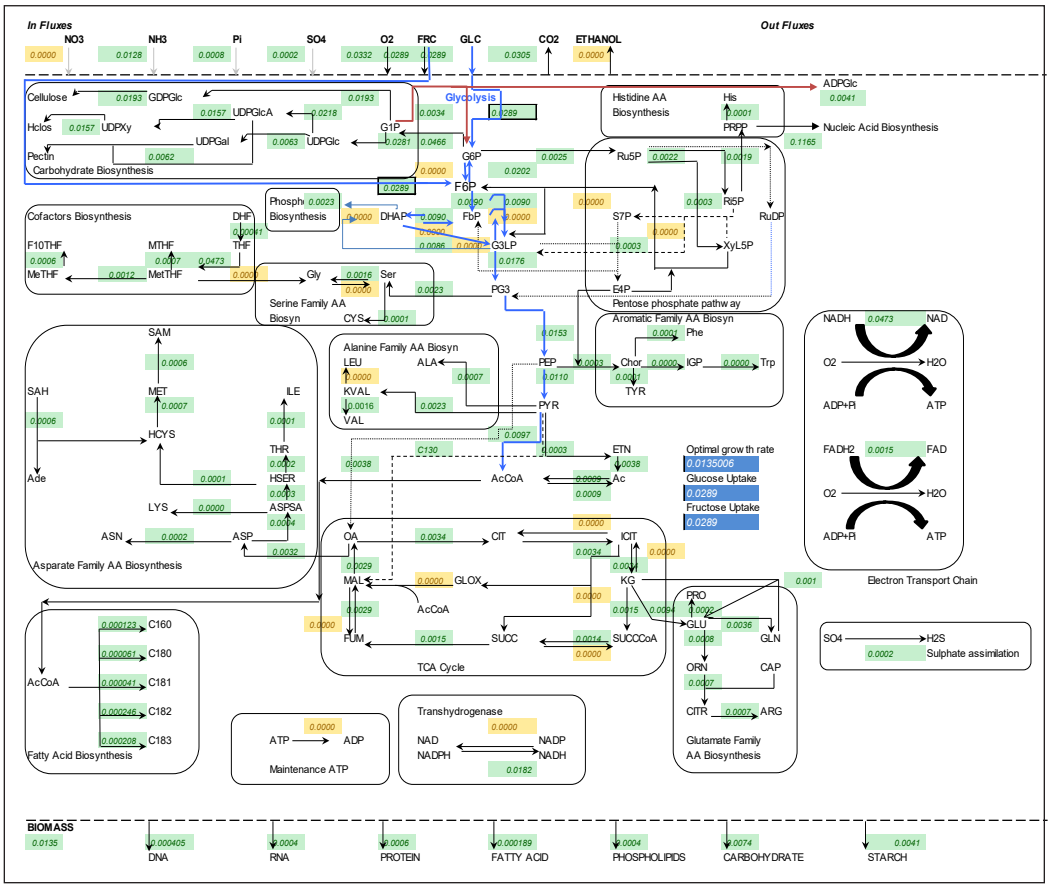

Figure 1. Metabolic flux map for rice cell suspension culture during 144 h of growth with maximization of starch and biomass production rate as the objective function

transport of metabolites between organelles or cell compartments. Starch biosynthesis is reported to occur in the chloroplast (Pfister & Zeeman, 2016), whereas in this study, the model considered all reactions to take place in the cytosol. Further reconstruction of the metabolic network in this study is required to optimize the starch production further. Although the starch produced in this study is lower compared to what has been reported, the whole mechanism of cell growth and metabolite synthesis is reflected in this model.

In addition, it was observed that the optimized specific growth rate values are higher compared to the experimental ones (Table 4). Therefore, it concluded that the cells were not growing at their maximum theoretical biological capacity from the supplied sugars. Nevertheless, this is expected since the experiments conducted by Lakshmanan et al. (2013) are not under optimized conditions. In their study, rice cells in suspension were grown under flooding and drought stresses to evaluate the effect on their metabolism. The overall trend of the optimized specific growth rate followed the experimental trend with the lowest value at 312 h due to the lowest total incoming carbon flux into the central metabolism.

Table 4
Carbon flux distribution during growth and starch optimization of cell suspension culture of rice

Time (h)	144	192	240	288	312
$\mu_{experimental}$ (h^{-1})	0.0052	0.0022	0.0038	0	0
$\mu_{optimized}$ (h^{-1})	0.0135	0.0028	0.0074	0.0035	0.0007
Total incoming carbon flux (mmole/g CDW h)	0.3468	0.0732	0.189	0.2892	0.0606
Carbon channeled into biomass (%)	84.4143	84.4143	84.4143	84.4143	84.4143
Carbon diverted to PPP (%)	3.2371	3.2371	3.2371	3.2371	3.2371
Carbon diverted to TCA cycle (%)	12.3487	12.3487	12.3487	12.3487	12.3487

Nonetheless, the overall flux distribution did not change significantly from one point of time to another (Table 4), which perhaps indicates that more constraints should be fed into the program, such as specific uptake rates for other nutrients and oxygen consumption, to see the variations in the carbon flux partitioning during the cell growth.

Specific Nutrient Uptake Rates

Nutrients are supplemented to the plants to ensure good growth and development. It is because the plant that lacks nutrients will not be able to complete its life cycle. For example, a seed may not be able to germinate, and a plant may not be able to develop roots and flowers properly. Moreover, the plant itself will often die upon the unavailability of nutrients. However, an excess amount of nutrients could also cause harm to plants. Thus, nutrient uptakes should be optimized to ensure the good growth of the plant. Besides, a higher biomass formation rate where starch is a part of it will result in a higher uptake rate of nutrients, which makes investigating the nutrient uptake rates important. In relation to that, different types of plants would have different nutrient uptake rates (Tony & McFarland, 2021).

In this study, due to the lack of experimental data on the specific uptake rates for ammonium, nitrate, phosphate, and sulfate, they were set as a free variable which GAMS determined upon optimization. It was observed that cells took up ammonium at the highest rate, followed by phosphate and sulfate (Table 5). It is correlated with a previous study on nutrient uptake in which rice took up nitrogen sources in different forms with ammonium at the highest rate, followed by phosphate (Masni & Wasli, 2019). Apart from that, the planted paddy preferred ammonium rather than nitrate as its main nitrogen source (Wang et al., 1993). Nitrogen is an essential macronutrient for

Table 5
Fluxes for the inorganic nutrient uptake in cell suspension culture of rice at 144 h of growth

Nutrient	Flux (mmole/g DW.h)
Ammonium	0.0128
Phosphate	0.0008
Sulfate	0.0002
Nitrate	0

the good growth of rice-cultivar as it increases plant height, panicle number, leaf size, spikelet number, and the number of filled spikelets, which largely determine the yield capacity of rice plant.

Sensitivity Analysis

In GAMS software, the marginal value of the intermediate metabolites and metabolic fluxes can be determined where it is information about the sensitivity of the optimal value to a change in the objective function. Specifically, in this study, sensitivity analysis allows the determination of significant metabolites that affect the set objective function in which it is represented in the form of marginal values.

Table 6 lists the metabolites that have the most significant effect on each objective function. Generally, both objective functions are highly affected by fatty acids, but they differ in terms of the amount of the marginal value, where marginal values for biomass maximization are higher than starch maximization. It is because starch is a part of biomass composition. Thus, when biomass maximization was set as the objective function, it meant maximization for all components of the biomass, not for the starch alone. Furthermore, although the composition of fatty acids in biomass (Table 1) is low, in the biosynthesis of 1 g fatty acids, 39 mmoles ATP, 44 mmoles AcCoA, and 72 mmoles of NADPH (based on the reconstructed stoichiometric metabolic model) are used which indicate that the energy intensiveness of fatty acids biosynthesis draining these precious resources for a small part of biomass formation (Puad, 2011).

Table 6
Sensitivity of the biomass and starch production rate for changes in various metabolites in terms of marginal values

Metabolite name	Marginal values (Biomass maximization as an objective function)	Marginal values (Starch maximization as an objective function)
Facids	-3.4322	-1.0537
Protein	-1.7020	-0.52257
Chydr	-1.5048	-0.4620
Stearic acid (C180)	-1.0081	-0.3095
Biomass (SS)	-1.0000	-

CONCLUSION

To conclude, the metabolic model proposed in this study can give exposure to the starch production mechanism in the plant cell culture even though the starch production predicted in this study is still considered to be lower compared to the whole plant. Nevertheless, it has been identified that the sugar uptake rates, specifically glucose, have a major impact on the starch production rate. Thus, it could be proposed that a high glucose uptake rate could contribute to high starch production. Due to that, in terms of the formulation of

the media, higher sugar concentrations should be supplied to achieve a high amount of starch production. However, the limitation of the amount of sugar that should be supplied requires further experimentation. Furthermore, additional constraints other than sugar uptake rates could give a different result because this study only relies on it as a constraint. Therefore, based this study's findings, further improvements should be made to develop a better model for the rice cultures, specifically in terms of the experimental data and the metabolic pathways. Lastly, supposed validation of the FBA results could be done through experimentation. In that case, the simulated results could be applied to the metabolic engineering of the plant to enhance starch production and even explain the interactions between pathways.

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ABBREVIATIONS

Ac	Acetate
Ade	Adenosine
ADP	Adenosine 5'-diphosphate
Ala	L-Alanine
AMP	Adenosine monophosphate
Arg	L-Arginine
Asn	L-Asparagine
Asp	L-Aspartate
ASPSA	Aspartate Semialdehyde
C160	Palmitic acid
C181	Oleic acid
C182	Linoleic acid
C183	Linoleneic acid
CaP	Carbamoyl phosphate
CDP	Cyitidine diphosphate
CDPDG	CDP-diacylglycerol
Chor	Chorismate
Cit	Citrate
Citr	Citrulline
CL	Cardiolipin
CMP	Cyitidine monophosphate
CO2	Carbon dioxide
CO2_exp	Exported carbon dioxide
CTP	Cytidine triphosphate
Cys	L-cysteine
dADP	deoxy-adenosine diphosphate
dCDP	deoxy-cytidine diphosphate
DG	diacylglycerol
DGDG	digalactosyl diacylglycerols
dGDP	deoxy-guanosine diphosphate
DHAP	dihydroxyacetone phosphate
DHF	7,8 Dihydrofolate
dTMP	deoxy-thymidine monophosphate
E4P	Erythrose 4-phosphate
Ethn	Ethanol
Ethn_exp	Exported ethanol
F10THF	N10-Formyl-THF
FbP	Fructose 1,6-biphosphate
FADH2	Flavine adenine dinucleotide
Frc_med	Fructose in medium
Frc	Fructose
Fum	Fumarate
GDP	Guanosine diphosphate
GDPGlc	GDP-glucose
Glc_med	Glucose in medium
Glc	Glucose
Gln	L-Glutamine
Glox	Gloxylate
Glu	L-Glutamate
Gly	Glycine
Gly3P	Glycerol-3-phosphate
GMP	Guanosine monophosphate
GTP	Guanosine triphosphate
H2S	Hydrogen sulfide
Hcellulose	Hemicellulose
Hcys	Homocysteine
His	Histidine
HSer	Homoserine
ICit	Isocitrate
IGP	Indoleglycerolphosphate
Ile	L-Isoleucine
ISMP	Inosinic acid (inosine monophosphate)
Ino	Inosol
KG	a-Ketoglutarate
Kval	Ketoisovalarate
Leu	Leucine
Lys	Lysine
Mal	Malate
Met	Methionine
MeTHF	N5-N10-methyl-THF
MetTHF	N5-N10-methylene-THF
MGDG	Monogalactosyl diacylglycerol
MTHF	N5-methyl-THF
MTR	methylthioribose
NAD_plus	Nicotineamide-adeninenucleotide
NADH	Nicotineamide-adeninenucleotide (reduced)
NADHm	Nicotineamide-adeninenucleotide mitochondrion (reduced)
NADP_plus	Nicotineamide-adeninenucleotide phosphate
NH3_imp	Imported ammonium
NH3	Ammonium
NO3_imp	Imported nitrate
NO3	Imported nitrate
OA	Oxaloacetate
OBJECTIVE	Objective function
Orn	Ornithine
Oxy_imp	Imported oxygen
Oxy	Oxygen
PA	Phosphatidic acid
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
Pect	Pectin
PEP	Phosphoenolpyruvate
PG	Phosphoglycol
Phe	L-phenylalanine
Pi_imp	Imported inorganic orthophosphate
Pi	Inorganic orthophosphate
PIno	Phosphatidylinositol
PPi	Inorganic pyrophosphate
PRAIC	5'-Phosphoribosyl-5-amino-4-imidazole carboxamide

Pro	L-Proline
PRPP	5-Phosphoribosyl-pyrophosphate
PS	Phosphatidylserine
Pyr	Pyruvate
Ri5P	Ribose 5-phosphate
Ru5P	Ribulose 5-phosphate
S7P	Sedoheptulose 7-phosphate
SAH	S-adenosyl-homocysteine
SAM	S-adenosyl-methionine
Ser	L-serine
SO4	Sulphate
Succ	Succinate
SuccCoA	Succinate Coenzyme A
THF	Tetrahydrofolate
Thr	L-Threonine
Trp	L-Tryptophan
Tyr	L-Tyrosine
UDP	Uridine diphosphate
UDPGal	UDP-Galactose
UDPGlc	UDP-Glucose
UDPGlcA	UDP-Glucuronic acid
UDPXy	UDP-Xylose
UMP	Uridine monophosphate
UTP	Uridine triphosphate
Val	L-Valine
Xyl5P	Xylulose 5-phosphate
Plipids	Phospholipids
dAMP	deoxy-adenosine monophosphate
dCMP	deoxy-cytidine monophosphate
dGMP	deoxy-guanosine monophosphate