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Flux balance analysis for ethylene formation in genetically engineered *Saccharomyces cerevisiae*

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Abstract

Biosynthesis of ethylene (ethene) is mainly performed by plants and some bacteria and fungi, *via* two distinct metabolic routes. Plants use two steps, starting with *S*-adenosylmethionine, while the ethylene-forming microbes perform an oxygen dependent reaction using 2-oxoglutarate and arginine. Introduction of these systems into *Saccharomyces cerevisiae* was studied *in silico*. The reactions were added to a metabolic network of yeast and flux over the two networks was optimized for maximal ethylene formation. The maximal ethylene yields obtained for the two systems were similar in the range of 7-8 mol ethylene/10 mol glucose. The microbial metabolic network was used for testing different strategies to increasing the ethylene formation. It was suggested that supplementation of exogenous proline, using a solely NAD-coupled glutamate dehydrogenase, and using glutamate as the nitrogen source, could increase the ethylene formation. Comparison of these *in silico* results with published experimental data for yeast expressing the microbial system confirmed an increased ethylene formation when changing nitrogen source from ammonium to glutamate. The theoretical analysis methods indicated a much higher maximal yield per glucose for ethylene than was experimentally observed. However, such high ethylene yields could only be obtained with a concomitant very high respiration (per glucose). Accordingly, when ethylene production was optimized under the additional constraint of restricted respiratory capacity (*i.e.* limited to experimentally measured values) the theoretical maximal ethylene yield was much lower at 0.2 mol/10 mol glucose, and closer to the experimentally observed values.

1 Introduction

Sustainable production systems independent of fossil resources have become highly topical in the light of an increasing green-house effect. To meet future demands for such products, large efforts have been made to use renewable feed stocks in biotechnological processes using cell cultivations. To widen the range of biotechnological products, metabolic engineering, *i.e.* introduction of foreign enzymes in a production organism, is an important technology. Often, mathematical analyses are used in metabolic engineering approaches to calculate maximal yield values for existing network structures and to test, *in silico*, how these yields are affected by the introduction of new enzymes to the network, *e.g.* [1-3].

An important group of materials whose production rely almost entirely on fossil fuels are synthetic polymers, including plastics such as polyethylene. Ethylene (the monomer from which plastic is produced) is currently the most highly produced organic compound [4]. Ethylene can also be produced biologically; it is an important plant hormone (for reviews see *e.g.* [5, 6]) and various microorganisms (*e.g.* mold and bacteria) are able to synthesize ethylene [7]. The ethylene synthesis pathways in plants and microorganisms are quite distinct. The ethylene forming enzymes of plants (1-aminocyclopropane 1-carboxylate (ACC) synthase and ACC oxidase) use *S*-adenosylmethionine as substrate, which in turn is formed by condensing methionine with the adenosyl group of ATP. In the first reaction of ethylene formation, catalysed by ACC synthase, methylthioadenosine is formed as a by-product and in the last step carbon dioxide and cyanide are by-products. The physiologically relevant ethylene formation of microorganisms is using 2-oxoglutarate as substrate and has been found in the plant pathogenic bacterium, *Pseudomonas syringae*, and the fungus *Penicillium digitatum*. In these two organisms, a single ethylene forming enzyme (EFE) catalyses a complex set of two oxygen dependent reactions, *i.e.* formation of

ethylene and succinate from 2-oxoglutarate and arginine [8], with concomitant formation of the by-products carbon dioxide, guanidine, and L-Δ^1-pyrroline-5-carboxylate (P5C).

The aims of this study were to analyze the metabolic effects of introducing ethylene-producing systems in the yeast *S. cerevisiae* and to find strategies for optimisation of ethylene formation. The intention was to start with the most simple case; ethylene forming reactions incorporated into central carbon metabolism without growth. The metabolic networks containing either the plant or the microbial system were analyzed with respect to theoretical maximal ethylene production yields. For the *in silico* model of the microbial system several strategies for optimisation were tested. The connection between ethylene formation and different metabolic pathways such as proline synthesis, glutamate catabolism, and respiratory activity were studied and all were shown to influence the ethylene formation. Furthermore, these *in silico* results were compared with previously published experimental data of a metabolically engineered yeast strain with EFE activity which was recalculated to allow comparison with the theoretical analyses.

2 Formulation and optimisation of stoichiometric networks

For the stoichiometric analysis, the reaction network of yeast central carbon metabolism and the two ethylene formation pathways as depicted in Figures 1 and 2 were used. These metabolic networks were constructed based on common general knowledge as found in standard references and databases, for instances the KEGG database (http://www.genome.jp/kegg/ligand.html), and the specific stoichiometries of ethylene formation [8, 9]. The intention in the construction was to add ethylene formation and formation and replenishment of precursors and cofactors needed for the respective systems to the central carbon metabolism (glycolysis and pentose phosphate pathway) but not taking

growth into account. Thus, for balancing cofactors, reactions such as ATPase, adenylate kinase, NADPH conversion to NADP, and respiration (NADH conversion coupled to ATP formation) were added. The plant ethylene enzymes ACC synthase and ACC oxidase connect to yeast metabolism via *S*-adenosylmethionine which is used as a substrate for ethylene synthesis and is formed from methionine in yeast. The by-product of ACC synthase, methylthioadenosine, is also a natural by-product of polyamine synthesis in yeast and can be regenerated to methionine in the methionine salvage pathway, which is to a large extent similar in all organisms [10]. In the first step of this salvage pathway, adenine is cleaved off and is subsequently together with phosphoribosepyrophosphate (PrPP) converted to AMP. In the last reaction of the salvage pathway 4-methylthio-2-oxobutyrate is transaminated to methionine. In yeast, the amino group donors for this reaction are mainly aromatic and branched-chain amino acids [11]. The regeneration of the donor amino acids is coupled to glutamate since this amino acid is used as amino donor during the biosynthesis of amino acids [12]. The 2-oxoglutarate formed from glutamate in the transaminations will be converted back by glutamate dehydrogenase using NADPH as cofactor. The source for both NADPH and PrPP is the pentose phosphate pathway using glucose-6-phosphate as substrate. In the ACC oxidase step, ascorbate is used as cofactor to form ethylene. The ascorbate/dehydroascorbate redox couple may be balanced with NADPH *via* the glutathione pool by a dehydroascorbic acid reductase, but such a reductase has so far not been identified in yeast. Thus, the performance of the pentose phosphate pathway will be crucial for an optimal ethylene production by providing both the NADPH and PrPP required. The microbial ethylene forming enzyme (EFE) connects to yeast metabolism at another site. EFE uses the citric acid cycle intermediate 2-oxoglutarate together with the amino acid arginine to form ethylene. By-products of the reaction are succinate (an intermediate in the citric acid cycle), guanidine, and P5C. Unfortunately, a

pathway for guanidine degradation or utilisation could not be identified. P5C is an intermediate in proline biosynthesis and yeast has enzymes which convert P5C to either proline or glutamate [13]. Thus, for the microbial system the network contains both the central metabolic parts (glycolysis, pentose phosphate pathway and citric acid cycle) as well as synthesis of arginine and proline. A notable feature of the network is the mitochondrial location of many reactions (*e.g.* arginine synthesis and glutamate formation from P5C). However, this was not taken into account in the stoichiometric analysis done here. In general, the network is highly dependent on redox metabolism since many reactions close to the ethylene formation via EFE are coupled to either NAD(H) or NADP(H).

Flux Balance Analysis (FBA, *e.g.* [14]), was used for calculation of the optimal flux distribution to maximize ethylene production. For each of the reaction networks a stoichiometry matrix was constructed. The matrix consisted for the plant system of 32 reactions and 29 independent variables and for the microbial system of 39 reactions and 34 independent variables. The concentrations of the following compounds were set as parameters in the plant network: glucose, CO_2, ethylene^{ext} (external ethylene), cyanide, formate, and sulphate, and in the microbial network: glucose, CO_2, ethylene^{ext}, PrPP, O_2, guanidine, and proline. The flux to ethylene was maximized under the constraint of steady state (*i.e.* all ordinary differential equations were set to zero), with a fixed influx rate. The solution is a flux ratio of the maximal attainable ethylene production per influx rate. As influx rate a combined action of glucose transport and phosphorylation was used: Glucose + ATP → Glucose-6-phosphate + ADP and fixed at a value of 10.

3 Results & Discussion

3.1 Ethylene formation by the plant enzymes

The stoichiometric network of yeast central carbon metabolism expanded with the ethylene synthesis pathway of plants and cofactor replenishment (Figure 1) was used in the metabolic network analysis. After optimizing for maximal ethylene formation a theoretical maximal yield of 7.6 mol ethylene/10 mol glucose was obtained. The analysis also confirmed that the important parts needed for proper functioning are the methionine salvage pathway (to regenerate methionine), the pentose phosphate pathway (to supply NADPH and PrPP), respiration (to supply ATP), and glycolysis (to supply ATP and pyruvate for citric acid cycle resulting in NADH formation used in respiration). Since 2 molecules of NADPH are needed for generation of glutamate and ascorbate and the pentosephosphate pathway yields 2 molecules of NADPH when 1 molecule of PrPP is formed, all the carbon coming into the pathway is channelled into ethylene and none returned to glycolysis. In the optimized network all carbon in ethylene is derived from PrPP and none via biosynthesis of methionine.

3.2 Ethylene formation by the microbial enzyme (EFE)

The metabolic network including the microbial ethylene formation by EFE (Figure 2) was used for the network analysis. In addition, since P5C is an intermediate in proline synthesis and P5C formed during ethylene production may be transferred to the pool of this amino acid we decided to fix the flux to proline formation at the level reflecting normal content of biomass, *i.e.* 30-40 mg/g protein [15]. Thus, using literature data [15, 16] the flux to proline may be estimated as 0.03-0.1 mol/10 mol glucose. Therefore, in the EFE network the flux towards proline formation from P5C was fixed at a low level of 0.1 mol/10 mol glucose. In a similar way as previously described for the plant system, an initial optimisation of the

microbial system was performed with the fixed formation of proline at 0.1 and with this system the theoretical maximal yield of ethylene formation was calculated to be 7.3 mol ethylene/10 mol glucose (Table 1). In such an optimised network there is no ATP limitation and the main function of the citric acid cycle is to provide carbon for ethylene formation. Thus, there is no need for 2-oxoglutarate dehydrogenase activity and succinate is formed solely as a by-product of ethylene formation.

3.3 Strategies for improving ethylene formation via the EFE system

Both the EFE system and the plant enzymes have been successfully expressed in *S. cerevisiae*. In our group an ethylene production of 147-210 μg/g dry weight cells/h by EFE-expressing yeast was recorded in aerobic batch cultures on glucose [17]. Lu et al [18] reported for the plant system in yeast a maximal ethylene production of 0.47 nl/10^8 cells/h. Using a conversion factor of $6.6 \cdot 10^{10}$ cells/g dry weight [19] and the molar gas volume equation, this production rate could be recalculated to 0.3 μg/g dry weight cells/h. Since, this value for the plant system is about 1000-fold lower than the corresponding value for the EFE system we continued to only do optimisation work for the EFE system. It might not come as a surprise that the EFE system with its bacterial origin (it is also found in fungi) performs better in yeast than the plant system. Another factor possibly contributing to achieving the low yield by the plant system may be that cyanide is released as a by-product. Since cyanide is known to be toxic and affect especially respiration of cells, a lower ethylene production may be expected and the plant system has thus a lower potential to be used in a production organism. Another cause for the low ethylene production may be a probable lack in yeast of a dehydroascorbic acid reductase, which is needed for co-factor regeneration.

As mentioned above many reactions connected to ethylene formation for the microbial system are redox reactions coupled to NAD(P) and the balancing of these redox cofactors may affect the ethylene production. Since arginine, one of the substrates, is formed from 2-oxoglutarate via glutamate (as part of the glutamate family of amino acids, *i.e.* glutamine, glutamate, arginine, lysine and proline [20]), the redox-cofactor specificity of glutamate dehydrogenase (Gdh) may be important in this respect. In the normal case during growth on ammonium as nitrogen source the NADP-coupled Gdh1 is used. The replenishment of NADPH is mainly done by the pentose phosphate pathway. But, this pathway gives a loss of carbon in the form of carbon dioxide. This indicates that one route to increase the ethylene yield is to change the cofactor dependency of Gdh from NADP to NAD. Indeed, running the network analysis with the cofactor exchange confirmed an increased ethylene formation (to 7.8 mol/10 mol) and a concomitant reduced flux through the pentose phosphate pathway, about 3 times from 5.5 to 2.0 mol/10 mol glucose. In yeast, there is also a NAD-Gdh present which is mainly expressed during growth with glutamate as nitrogen source and our model suggests that deleting the two NADP-dependent isoenzymes, Gdh1 and Gdh3 [21], may be a strategy for increased ethylene formation. Similarly, *in silico* modelling has suggested such a shift in cofactor specificity of Gdh for optimal sesquiterpene formation in yeast [2]. This prediction was also verified experimentally by overexpression of Gdh2 in a strain carrying a *GDH1* deletion. For our EFE-system the *in silico* model predicted a 7% increase in the ethylene production using such an experimental strategy (Table 1).

The formation of proline was also allowed to vary in order to achieve an optimal ethylene formation. The flux to proline from P5C was found to always convert to zero when optimising the ethylene flux and thus a conversion of P5C to glutamate is advantageous. In this situation the ethylene yield increased to 12 mol/10 mol glucose (the Gdh reaction was

coupled with NADP). Thus, one good strategy to increase the ethylene formation may be to add an exogenous source of proline.

3.4 Ethylene formation by the microbial system using different nitrogen sources

Another way to increase ethylene formation is to provide the precursor 2-oxoglutarate for ethylene formation. To achieve this, glutamate may be used as the sole nitrogen source since during such cultivations it has been found that large amounts of 2-oxoglutarate is formed [15, 16, 20]. An additional advantage of using glutamate is that the other precursor arginine is also formed from glutamate [12]. The metabolic network was therefore updated to allow for glutamate inflow. When using glutamate as sole nitrogen source a consumption of 0.12 g glutamate/g glucose (*i.e.* 1.5 mol/10 mol) has been reported during aerobic growth [16, 22]. However, some of the glutamate will be directly incorporated into biomass as amino acids of the glutamate family [20] and an inflow of glutamate corresponding to these could be estimated as 0.2 mol glutamate/10 mol glucose using literature data [15, 16, 20]. Thus, an inflow of glutamate was set as 1.3 mol/10 mol glucose, glutamate dehydrogenase reaction was coupled to NAD, and the concentration of external glutamate was additionally set as a parameter. The results for the optimisation of this network showed an increased ethylene formation from 7.8 to 8.7 mol ethylene/10 mol glucose when changing nitrogen source from ammonium to glutamate (Figure 2 and Table 1).

3.5 Recalculation of experimental data of ethylene production via the EFE system

In our published experiments with a bacterial EFE expressed in yeast, cells were grown in bioreactor cultivations using either ammonium or glutamate as nitrogen source [17]. In line with our modelling results, the formation of ethylene was greatly increased with glutamate compared to using ammonium as nitrogen source. The yields reported in this study were

890±160 µg ethylene/g glucose (*i.e.* 57±10 mmol ethylene/10 mol glucose, SD given as ± value) with glutamate and 320±90 µg/g (*i.e.* 21±6 mmol/10 mol) when using ammonium. However, these yields were obtained for complete aerobic batch cultivations, *i.e.* including growth on glucose as well as ethanol and thus these data were recalculated to only take into account the glucose phase (Table 2) to allow comparison with the *in silico* results. Also for these recalculated data it was obvious that glutamate is a superior nitrogen source compared to ammonium in terms of ethylene production. The data also showed a similar ethanol formation and a slightly decreased biomass formation using glutamate as the nitrogen source. The maximal specific growth rate was decreased by 39% in the glutamate cultures which is in accordance with previous results for anaerobic cultures with ammonium or glutamate for which a 27% reduction in growth rate was found [15].

The EFE system has also been expressed in other hosts; *e.g.* the fungi *Trichoderma viride* which produced ethylene at a rate of 4.1 mg/(g dry weight h) [23], which is similar to the yeast system. However, recently Wang et al [24] engineered *Pseudomomas putida*, for ethylene production to achieve production rates of 80.2 mg/(g dry weight h).

3.6 Possible explanations for the low experimental ethylene production yields

Upon expression of the EFE system in yeast, ethylene formation was observed and using glutamate as nitrogen source resulted in a more than doubling of the ethylene production rate as compared to using ammonium. However, these yields were much lower than the theoretically obtainable yields as calculated in the stoichiometric analysis. Several reasons might account for this discrepancy: Firstly, the stoichiometric network is not complete in that it does not include biomass formation. Therefore, in order to achieve a better comparison of the theoretical ethylene yields, the experimental yields were adjusted to show yields on glucose subtracting the part going to ethanol or biomass (Table 2).

However, even when corrected for the carbon going to these large "by-products" only a very small part of the glucose influx was going to ethylene production (about 1.1 and 0.8% for the glutamate and ammonium cultures, respectively). This is in sharp contrast to the theoretical 73% yield given by the model for the ideal case.

A second and more important reason for the discrepancy between the theoretical obtainable yields and the experimentally observed yields is that most likely the stoichiometry of the network is not limiting the ethylene production yields but rather the kinetics of the reactions steps in the network and/or that effects from the regulation of protein expression/activity limit the flux through some steps. The effect can be found at two sites; the ethylene formation itself and in the metabolism providing necessary cofactors and precursors. Firstly, the inserted EFE enzyme may not exhibit high enough activity to allow high ethylene production. This is supported by the recent work of Wang et al [24] who found that inserting additional copies of the *EFE* gene increased the enzyme activity and the ethylene formation in metabolically engineered *Pseudomonas putida*. However, we expressed the EFE enzyme under the control of a *TPI1* promotor on a multicopy plasmid, and an expression defect is therefore not likely. Insufficient enzyme activity is more likely to be caused by post-transcriptional/post-translational effects. Secondly, in all the optimized networks a high respiratory activity of 50 mol NADH/10 mol glucose or more, was found, which corresponds to 25 mol O_2/10 mol glucose. However, respiration in *S. cerevisiae* is glucose-repressed and such high respiration rates are never observed. A typical specific respiration rate during a fully aerobic batch cultivation on glucose has been observed to be in the range of 1-3 mmol O_2/g cells h [25]. This range can thus be used to estimate a yield per glucose of respiration in our cultures by normalising this flux range with the specific glucose consumption rate (mmol glucose/g cells h). The specific glucose consumption rates determined in our cultures were approximately 11 mmol/g h irrespective of the type of

nitrogen source (Table 2), resulting in a respiration yield of 1-3 mol O_2/10 mol glucose or 2-6 mol NADH/10 mol glucose for the typical range of respiration given above. Thus, physiological relevant respiration levels are at least ten times lower than the results from the stoichiometic analyses and a low respiration capacity may be part of the explanation for the low ethylene formation found experimentally. An optimization of the metabolic network for the ammonium case was performed by constraining the respiration to a fixed level of 8 mol NADH/10 mol of glucose (which was the lowest level giving a stable solution of the metabolic network, but it is still significantly higher than the values discussed above). For this case the ethylene formation was drastically reduced to 0.2 mol/10 mol glucose (Table 1). Thus, with a more realistic additional constraint on respiratory activity a much lower theoretically obtainable ethylene yield was calculated and the metabolically engineered yeast strain achieved 41% of this maximum ethylene yield.

4 Conclusions

In this work, the expression of ethylene producing systems in yeast was studied *in silico* by flux balance analysis of simplified systems with ethylene forming reactions added to central carbon metabolism excluding growth. For the plant system the pentose phosphate pathway, citric acid cycle, and the methionine salvage pathway were found to be important for a maximal ethylene yield. The microbial EFE-system was more extensively studied and used for identification of targets for optimising ethylene production. It was found that the co-factor specificity of glutamate dehydrogenase was important; activity coupled with NAD instead of NADP increased ethylene formation. This situation could be achieved by deleting the NADP-coupled Gdh1p combined with overexpression of the NAD-coupled Gdh2, which however remains to be tested. The other main identified options for obtaining an increased ethylene formation were to add proline to the medium or use glutamate as

nitrogen source. Furthermore, the results for the microbial system were compared with recalculated published experimental data and the relevance of the prediction that changing nitrogen source to glutamate will increase ethylene formation was verified by the experiments. However, the ethylene formation detected experimentally was far below the theoretical maximum and one explanation for this discrepancy was the limited respiratory capacity in *S. cerevisiae* during aerobic glucose growth. In agreement with this, network optimization with a limited respiration largely decreased the ethylene formation. Consequently, the experimental ethylene formation (adjusted for carbon going to ethanol and biomass) was 41% of the theoretical formation when limiting the respiration. Thus, the current model, even with all included simplifications can come close to predicting experimentally determined ethylene yields. The next step will be to use an extended model including growth and subcellular compartmentation, preferably a genome wide model with experimental data for respiration and formation of biomass, ethanol and other products included in the flux balance analysis, as published [26].

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6 References

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Tables including legend

Table 1. Results from the flux balance analyses for the plant and microbial systems at different conditions. Gdh is glutamate dehydrogenase coupled either with NADP or NAD as indicated.

Model condition	Specific constrains	Ethylene yield (mol/10 mol glucose)
Plant model		7.6
Microbial model, fluxes ≥0	Proline flux = 0.1 mol/10 mol, NADP-Gdh reversible	7.3
	Proline flux=0.1 mol/10 mol, NAD-Gdh reversible	7.8
	Proline flux ≥ 0, NADP-GDH reversible	12
Microbial model, fluxes reversible[1], glutamate inflow incorporated, NAD-Gdh	Glutamate influx = 0	7.8
	Glutamate influx = 1.3 mol/10 mol	8.7
	Glutamate influx = 0, respiration ≤ 8	0.2

[1]Except for formation of PrPP and proline, reactions catalysed by glucose-6-phosphate dehydrogenase, 2-oxoglutarate dehydrogenase, and ATPase, and conversion of NADPH to NADP, which were constrained to be ≥0

Table 2. Maximal specific growth rate, μ_{max}, specific glucose consumption rate (mmol/g dry weight of cells/h), and product yields[a], Y, (mol produced/10 mol glucose consumed) during the glucose consumption phase in aerobic batch cultures, calculated using raw data from reference [17]. The yield for ethylene is also adjusted to represent the carbon of glucose not going to formation of ethanol (with accompanied CO_2 evolution) and biomass, $Y_{ethylene}^{corr}$. Triplicate cultivations were done and means ± standard deviation are presented.

Type of nitrogen source	μ_{max} (h^{-1})	Glucose consumption rate (mmol/g cells/h)	$Y_{ethanol}$ (mol/10 mol)	$Y_{biomass}$ (C-mol/10 mol)	$Y_{ethylene}$ (mol/10 mol)	$Y_{ethylene}^{corr}$ (mol/10 mol)
Ammonium	0.41±0.03	12.6±2.5	14.6±1.5	10.3±0.8	0.0082±0.0021	0.082
Glutamate	0.25±0.01	10.0±2.2	14.1±0.3	7.5±2.6	0.0189±0.0084	0.111

[a]yields during the glucose phase were calculated from the raw data of reference [17] as the negative slope of either ethanol, biomass or ethylene concentrations to the glucose concentrations by linear regression.

Figure legends

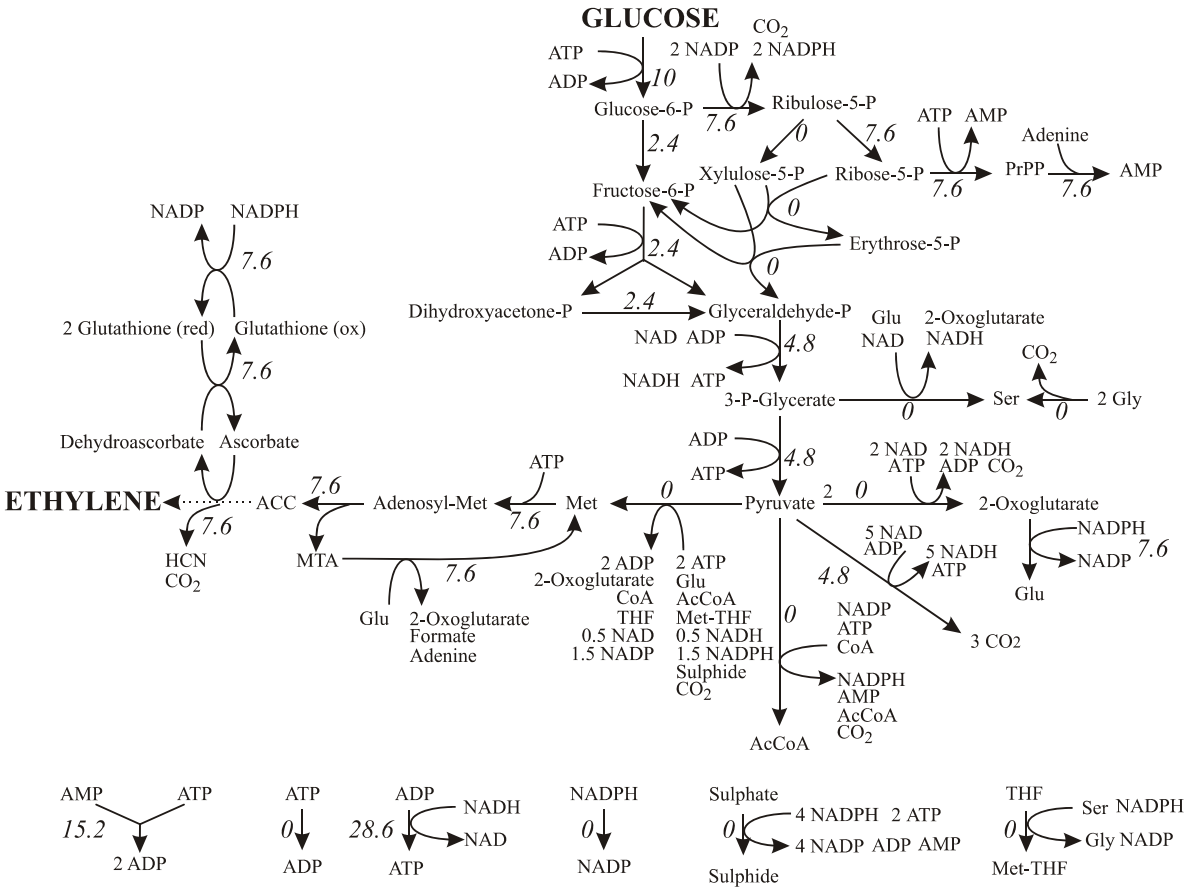

Figure 1. The stoichiometric network of ethylene formation used in the mathematical analyses for the plant ethylene formation system. Abbreviations used are MTA-methylthioadenosine, (Met-)THF-, (5-methyl)-tetrahydrofolate, AcCoA-acetyl coenzyme A, standard abbreviations of amino acids, or as indicated in the text. The reaction with a dotted arrow was optimized in the analysis. The numbers in italic are the result of the optimization with reactions ATP to ADP and NADPH to NADP forced to have a rate ≥0 and the others set free. The following compounds were set as parameters: glucose, CO_2, ethylene, HCN, formate, and sulphate.

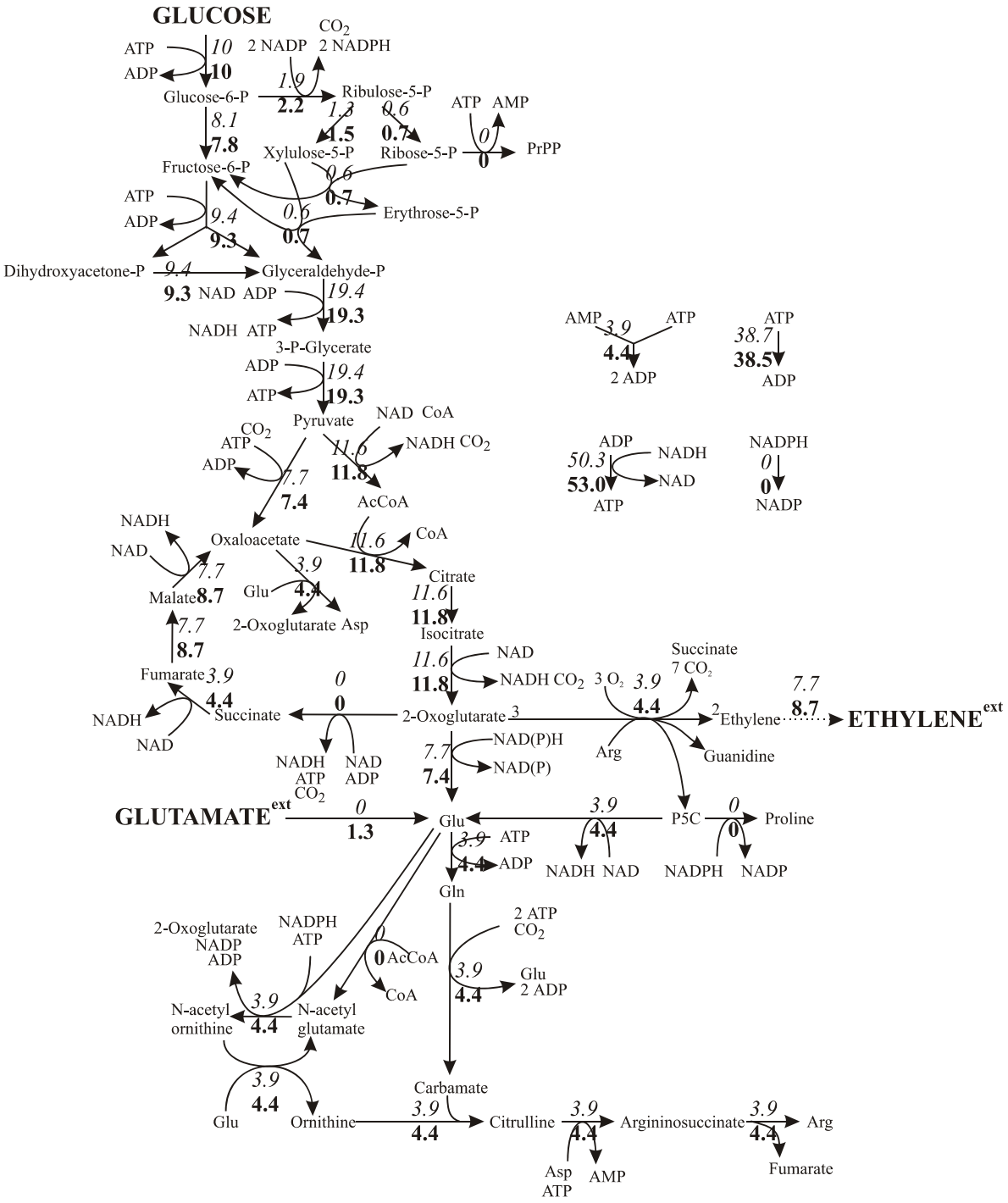

Figure 2. The stoichiometric network of ethylene formation used in the mathematical analyses for the microbial ethylene formation system.

Abbreviations used are, AcCoA-acetyl coenzyme A, standard abbreviations of amino acids, or as indicated in the text. The reaction with a dotted arrow was optimized in the analysis and the numbers are the results of the optimization comparing the use of different nitrogen sources, in italics using ammonium and in bold using glutamate as nitrogen source.