

Investigation of the metabolic changes in the adaptive evolution of  
*S. cerevisiae* strains by using genome-scale metabolic models

by

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## 1. INTRODUCTION

Evolution has been intensively studied since the publication of *On the Origin of Species* in order to untie the mysteries behind fascinating machineries within living systems. In a broader sense, most of the studies on evolutionary biology have two main goals: (1) To document history of life through evolutionary point of view, and (2) to understand causal mechanisms responsible for the biological diversity that we have on Earth [1, 2]. In addition to these goals, the idea of using pre-existing living systems as cell factories for industrial purposes gained close attention in the last decades [3].

Evolutionary engineering studies, adaptive laboratory evolution in particular, refers to the experiments in which the environmental conditions are altered gradually to obtain adapted populations in the laboratories [4]. Accumulation of mutations obtained due to environmental alterations through generations, where the favored individuals (mainly the ones with increased fitness) are selected to become parents of the next generation, result a population with advantageous traits compared to starting population. Since one of the main challenges in the evolutionary engineering field is being able to analyze experimental to answer fundamental questions, adaptive evolution studies usually require interdisciplinary research in order to answer fundamental questions. What has changed in the cells through generations? What are the genetic basis for adaptation? Can we evolve any organism to any condition? If so, how? These questions and many more are asked everyday, and the corresponding answers potentially give rise to more questions.

The purpose of this thesis is to enlighten metabolic changes in the adaptation of the yeast *S. cerevisiae* using computational methods, specifically genome-scale metabolic models. Evolved yeast strains (such as ethanol tolerant, long-lived, multi-stress resistant strains) are going to be analyzed comparatively to the unevolved strains using intracellular flux distributions obtained with the integration of transcriptomics data. This study will also contribute to the global understanding of metabolic regulations in yeast, and will be further expandable into metabolic engineering studies.

## 2. THEORETICAL BACKGROUND

### 2.1. Adaptive Laboratory Evolution

Since the very first laboratory evolution experiment was published in the late 19th century by William Dallinger, technological advancements allowed researchers to employ fully-controlled experiments for strain engineering to achieve desired traits [5]. Challenges in the experimental design of evolutionary studies, such as maintenance of the generations, controlling the environment, and feasibility to perform data analysis make the use of microorganisms in evolutionary studies more suited, especially *Escherichia coli* and *Saccharomyces cerevisiae* given their extensive characterization [6].

Commonly used approaches of adaptive laboratory evolution (ALE) includes chemostat cultures and serial batch or colony transfers (Figure 2.1). Serial transfer experiments in which the initial population is aliquoted and transferred into a new medium are simpler and cheaper to set up, however the possibility of genetic drift is higher due to random sampling of the population. On the other hand, continuous systems such as chemostat experiments in bioreactors have advantages on maintaining constant growth rates and population sizes, but with increasing experimental cost [7].

In addition to the methodological choice, decision of the selection criteria and the time span for the experiments are critical factors in ALE [5]. Growth rates, survivability in stressful environmental conditions and biomass yields are the most common fitness criteria for a population to be selected. Throughout the selection process, reaching  $>500$  generations may take a few weeks or a few months depending on the organism, therefore, detailed planning is essential [9].

Capturing genomic changes in the dynamic evolution process is the main goal of an ALE study, therefore frequent data collection in longitudinal manner is a must to unveil molecular details. Next-generation technologies empower researchers to catch even single-nucleotide mutations through genome sequencing and also provide

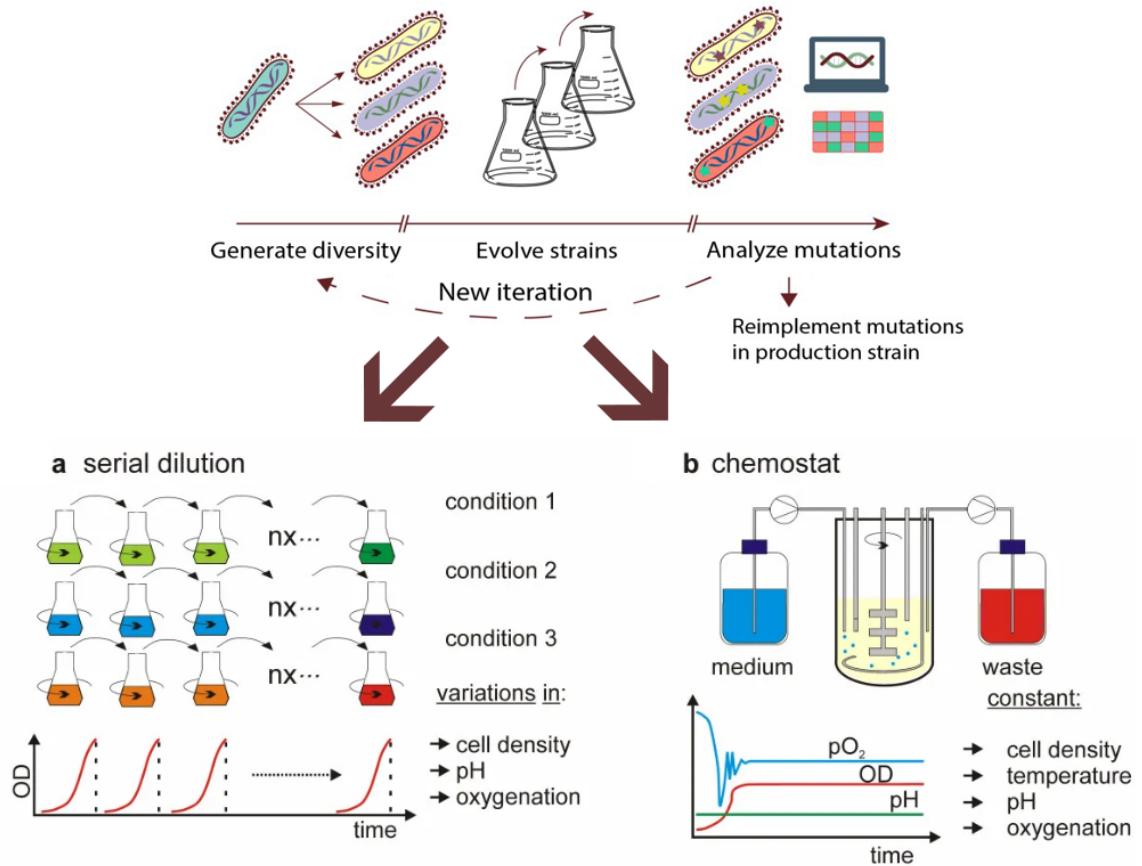


Figure 2.1. ALE methods and workflow. Figure is taken from [5] and [8] and will be redrawn altogether.

an understanding on broader regulatory changes through gene expression profiles [9]. Focus of the studying the differences between evolved and unevolved strains may be on the individual protein level or as a system-level trait. Interpreting the system as a network enables verious ways to investigate causality behind adaptive mechanisms and dynamics of evolution [10, 11].

## 2.2. Systems Biology

With the increasing availability of the computational tools and the development of high throughput techniques in the omics field, systems biology has shown a strong emergence in the last few years as a key multi-disciplinary field for integrating the multi-layer complexity of biological systems, particularly in the areas of transcriptomics,

proteomics, metabolomics and fluxomics [12]. This amount of available data allows researchers to investigate molecular cell processes in a large scales, applying theoretical, experimental and computational methods.

Biological systems based on complex interactions between various molecular components. The relations between these components are often obey nonlinear kinetics, for example, most of the reactions are regulated by one or more feedback or feed-forward loops with incomprehensible behaviours. When considered, cell structure and compartmentalization are also often introduce complexities to the unexpected behavior of the entire biological system [13]. Mathematical modeling with these factors taken into consideration is used as a general approach to encompass existing knowledge in biological systems, and to gather information by analyzing these models to acquire a better understanding [14].

A mathematical model of a cell can be approached by two different approaches in either a bottom-up or top-down directionality (Figure 2.2) [15, 16]. Top-down approach is an experimental oriented approach, it starts from the whole picture and aims to characterize biological mechanisms closer to the smaller parts and their interactions in the network. In the bottom-up approach, collected data from biological knowledge is used as a starting point, a subsystem is generated to deduce the functional properties of smaller points in the network. Combination of the pathway level models (bottom-up) into a model for the entire system level (top-down) is the ultimate goal in the systems biology therefore these approaches are complementary.

### 2.2.1. Metabolic Networks

In the context of systems biology, metabolic network reconstructions have become a common interest for the researchers over the past 20 years [17]. Organism-specific metabolic network analyses allow scientists to design experiments and even obtain beforehand predictions. These networks are the main sources of the mathematical models which can simulate metabolic fluxes reflecting the experimental reality [18].

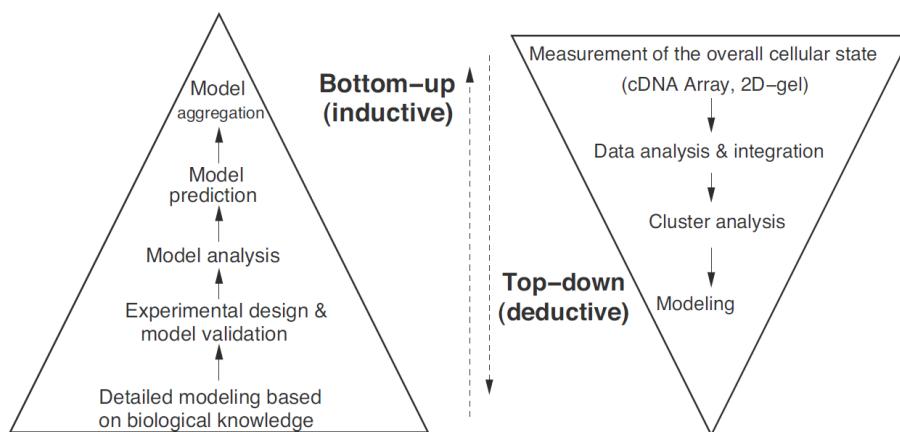


Figure 2.2. Systems biology approaches. Left: Bottom-up approach. Right: Top-down approach. Figure is taken from [14].

Before the improvement of genome sequencing or annotation technologies, initial core metabolic networks were based on the accessible information of biochemical pathways [19] [20]. In the last decade, larger genome-scale metabolic models (GSMMs) have been able to be developed rapidly with the help of databases for annotated genomes, providing information on substrates and products of each enzyme and each bioreaction [21]. Growing biochemical databases provide automatization processes for the metabolic network reconstructions. As a result, genome-scale metabolic networks are available today for almost all organisms with an annotated genome available in the literature [22, 23]. From the first genome-scale metabolic model of *Escherichia coli* to other organisms, the steps required for GSMM development remained the same regardless of the biological diversity.

A generally applicable protocol is defined by the Palsson group [17, 21] for the reconstruction of biochemical networks described in the Figure 2.3 [24]. Briefly, genomic data for the biochemical reactions of an organism are identified from the databases, such as NCBI, DDBJ and EMBL-EBI. Extraction and processing of the gene-protein-reaction relationship (GPR) of the genomic data results a draft reconstruction. GPR associations in the draft model should be reviewed by the researchers and manually curated if the identifying process is achieved with the help of automated computational algorithms [22].

Since the genomic data is the least representative of the biological phenotypes, available transcriptomic, proteomic, metabolomic and/or subcellular localization data are also used to further curate the model. Once the final metabolic network is reconstructed with bibliographic information, it is translated into a mathematical model.

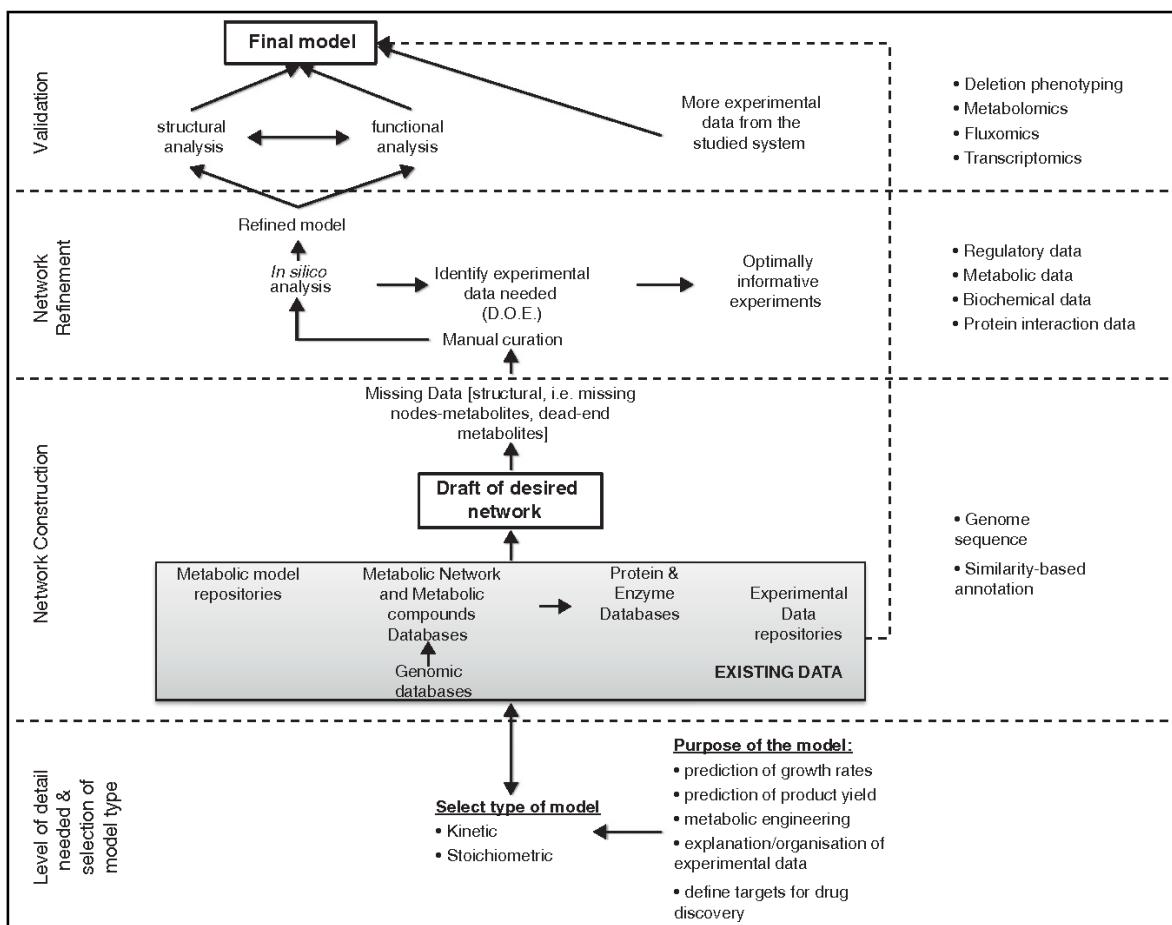


Figure 2.3. Overview of metabolic network reconstruction protocol. Figure is taken from [24].

Once a metabolic network is reconstructed, a rational link between a genome sequence, the proteins encoded in the genome, and the reactions catalyzed by the proteins allowing to investigate the relationships between genotype and phenotype is achieved [25]. As the final step, GSMM needs to be validated by the new experimental data sets. GSMM validation process for various experimental conditions require detailed cultivation data from experiments. For example, information on the biomass composition of the specific organism leads more accurate biomass equation in the model, that is one

of the key factors in the GSMM optimization and validation [26]. Even though multiple steps in the GSMM reconstruction can be achieved with the automated softwares available, it is usually necessary to curate the obtained model manually.

Approaches for analyzing metabolic networks are mainly categorized as dynamic or structural approaches. Even though the former is promising more realistic approach, its implementation in the literature is obstructed due to the unavailability of kinetic parameters for the majority of enzymes within a metabolic network [27, 28]. Because of the lack of kinetic parameters, structural metabolic modeling has been widely used for analyzing cellular metabolism at a steady-state assumption as a kind of snapshots taken at specific times.

GSMMs are one of the most useful tools in systems biology, especially in metabolic engineering studies [29]. In 1998, with the publication of *Metabolic Engineering: Principles and Methodologies*, the term metabolic engineering is defined as the optimization of natural processes within cells to increase the production of certain substances [30]. Hence, studies of metabolic engineering can be considered as genetic engineering in strain development. However, while metabolic engineering manipulates strains by altering flux distributions in the pathways; genetic engineering modificates specific genes, proteins and/or enzymes of interest [31]. Although GSMMs are mainly used in metabolic engineering strategies, other applications both for descriptive and predictive purposes can be found in the literature [32].

The ultimate goal of the GSMM reconstruction is to predict flux distribution profiles as close *in silico* as they are *in vivo*. Hence, GSMMs are in continuous research to improve predictability of organism-specific models.

### **2.2.2. Mathematical Representation of Metabolic Networks**

Basic applications of metabolic network modelling, although its framework is developed by engineers or mathematicians, is used by biologists with various mathematical backgrounds [33]. In order to speak with one voice, fundamental concepts of

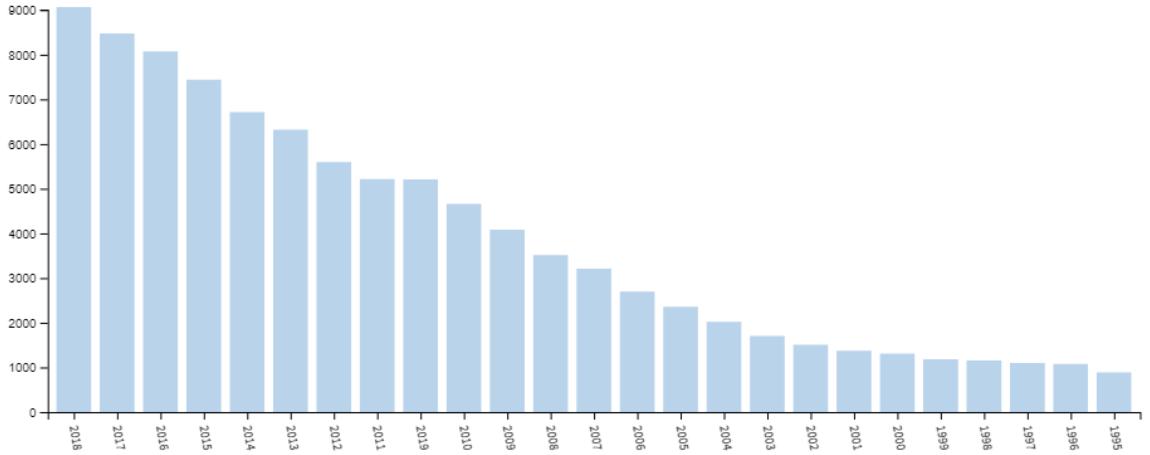


Figure 2.4. Web of Science article counts on "metabolic model"

mathematical representation of metabolic networks in GSMM reconstructions will be provided in this section.

One can propose a steady-state model for the correlation between the metabolites exist in the network. This model claims that the production and consumption of a metabolites must balance each other [34]. For example, consider the metabolite "A" in the toy network in Figure 2.5. It can be taken into cell with the rate of  $b_1$ , and can be converted into B or C with the rates of  $v_1$  and  $v_2$  respectively; at the same time, metabolite C can be converted into A with the rate of  $v_3$ . Note that enzyme kinetics are not considered and these equations are formed only considering mass balances.

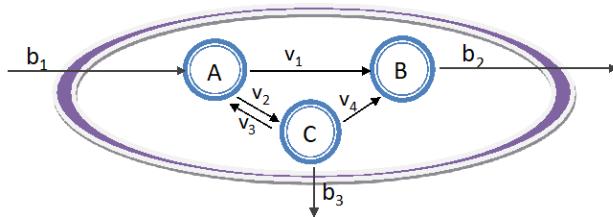


Figure 2.5. Toy network for steady-state assumption (will be redrawn).

If we accept the steady-state assumption, and since we know all the possible reactions passing through metabolite A, we can write following differential equation

$$\frac{d(A)}{dt} = -v_1 - v_2 + v_3 + b_1 = 0$$

meaning that production rate of metabolite A is equal to its consumption rate. In other words, there will be no accumulation of metabolite A in the cell. Considering we have 3 metabolites and 7 reactions, this representation of reactions can be written as a system of linear equations:

$$\begin{cases} \frac{d[A]}{dt} = -v_1 - v_2 + v_3 + b_1 \\ \frac{d[B]}{dt} = v_1 + v_4 - b_2 \\ \frac{d[C]}{dt} = v_2 - v_3 - v_4 - b_3 \end{cases} \rightarrow \begin{bmatrix} -1 & -1 & 1 & 0 & 1 & 0 & 0 \\ 1 & 0 & 0 & 1 & 0 & -1 & 0 \\ 0 & 1 & -1 & -1 & 0 & 0 & -1 \end{bmatrix} \begin{bmatrix} v_1 \\ v_2 \\ v_3 \\ v_4 \\ b_1 \\ b_2 \\ b_3 \end{bmatrix} = \begin{bmatrix} 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \end{bmatrix}$$

$$S \cdot v = 0$$

In the above equation, a matrix "S" appears. S matrix consists of stoichiometric coefficients of the reactions. A single column of S matrix, representing a single reaction, provides information about the connections between the metabolites participating in that reaction; and a single row of S matrix, representing a metabolite, provides information about the connection of all the reactions in which that metabolite participates.

Next to S matrix a vector "v" appears. Vector "v" is called the flux vector, and it contains the change in concentration of metabolites. In other words, flux vector represents the flow rates or "fluxes" of each metabolite over time.

The solution space of S matrix, in the steady-state assumption, is generated by the null space base vectors of S. Studying on the null space vectors, we can define dead-end reactions (zero rows which mean reactions cannot carry flux), enzyme subsets (rows of scalar multiplies of each other, possibly chain reactions) and independent components (diagonal block structures in the null space, reactions that are independent from the network). Due to the large number of reactions in biological networks, the system is underdetermined and have multiple solutions in a convex flux cone space, referring to multiple steady-states of the cell.

### 2.2.3. Constraint-Based Modelling

As explained in the previous section, solution of a metabolic network is not unique, meaning that the model can be found in multiple states of flux distributions. In order to obtain more biologically relevant solutions,  $v$  vector can be calculated within a set of constraints [35]. The rationale behind constraining the flux vector is that biological systems must obey general principles in nature, such as basic rules of chemistry and the laws of thermodynamics.

In terms of physico-chemical properties of a biological system, mass and charge balance must be conserved. These conservation laws are given into mathematical problem as "hard" constraints since these rules are inviolable [36]. Reaction reversibility is a hard constraint, determined by the laws of thermodynamics. Some reactions are irreversible by nature under certain conditions. These reactions are mathematically bounded in the flux vector, so they can have only positive ( $v_i \geq 0$ , forward directionality) or negative ( $v_i \leq 0$ , backwards directionality) values. Depending on the methodology, by reversing the equations of backwards-directed reactions, and splitting the reversible reactions into two forward and reverse reactions, one can obtain a vector  $v$  where each element is greater than or equal to 0.

Since the experiments are carried out in a defined medium, environmental constraints must be added in silico simulations as well. These constraints include nutrient availability (such as carbon, nitrogen or oxygen sources), the pH value, temperature, or any other experiment-specific conditions. Additionally, spatial constraints can be given to the system to limit substrate and enzyme availability for a specific compartment, especially for the metabolites which are transferred between compartments by facilitated diffusion.

More recently, regulatory constraints have started to be used to simulate experimental observations on cellular regulatory mechanisms, such as variable amounts of gene products (transcriptional, translational regulations) and their activities (enzymatic regulations). Integration of omics data, mainly transcriptome data, is used to capture

these regulations in silico.

### **2.3. *Saccharomyces cerevisiae***

The species "yeast" includes a range of eukaryotic single-celled microorganisms, although it is commonly used to describe *Saccharomyces cerevisiae*. Also known as the baker's yeast, *S. cerevisiae* is one of the extensively used microorganisms for alcoholic fermentation of beverages, bio-ethanol production, and processing various foods since ancient times [37]. It was the first eukaryotic organism whose genome was fully sequenced and annotated [38], and besides its benefits in the industry, it is used as a model system for other eukaryotic cells including humans [39, 40].

#### **2.3.1. Central Carbon Metabolism of *S. cerevisiae***

From the end of the eighteenth century, mainly after the fermentation is defined as "respiration without oxygen", the metabolism of *S. cerevisiae* has been studied extensively [41, 42]. Its capability to produce ethanol is one of the most characterized microbial processes due to industrial utilization.

The set of anabolic and catabolic reactions in the cell are referred as the metabolism. A schematic representation of the central carbon metabolism in *S. cerevisiae* can be found in Figure 2.6. Glycolysis, pentose-phosphate pathway (PPP), tricarboxylic acid cycle (TCA) or Krebs cycle, the glyoxylate cycle and the electron transport chain are the main pathways in central carbon metabolism.

In this section, there will be subsections on all biological pathways individually (explaining each in detail -probably referencing Lehninger biochemistry-) especially NAD regulation, fermentation (crabtree effect, industrial applications, bio-ethanol production) etc. Maybe also regulation strategies in cells (feedback/feedforward loops with figures)

### 2.3.2. Adaptive Evolution Studies on *S. cerevisiae* (Palsson 2015)

### 2.3.3. Metabolic Models of *S. cerevisiae*

After the first *S. cerevisiae* genome sequence is published, the first cDNA spotted microarray exploring metabolic gene regulation in 1997 [44], and the first commercial platform for oligonucleotide microarray data (Affymetrix) to investigate cellular regulations were reported in 1998 [45]. Existing genome data is integrated with the extensive annotation based on microarray data and biochemical knowledge from literature, leading of the publication of the first GSMM of *S. cerevisiae* in 2013 [46]. More in this section, there will be review on: Genome-scale modeling of yeast: chronology, applications and critical perspectives [47]

### 2.3.4. Applications of *S. cerevisiae* GSMMs

Literature review on the applications will be added.

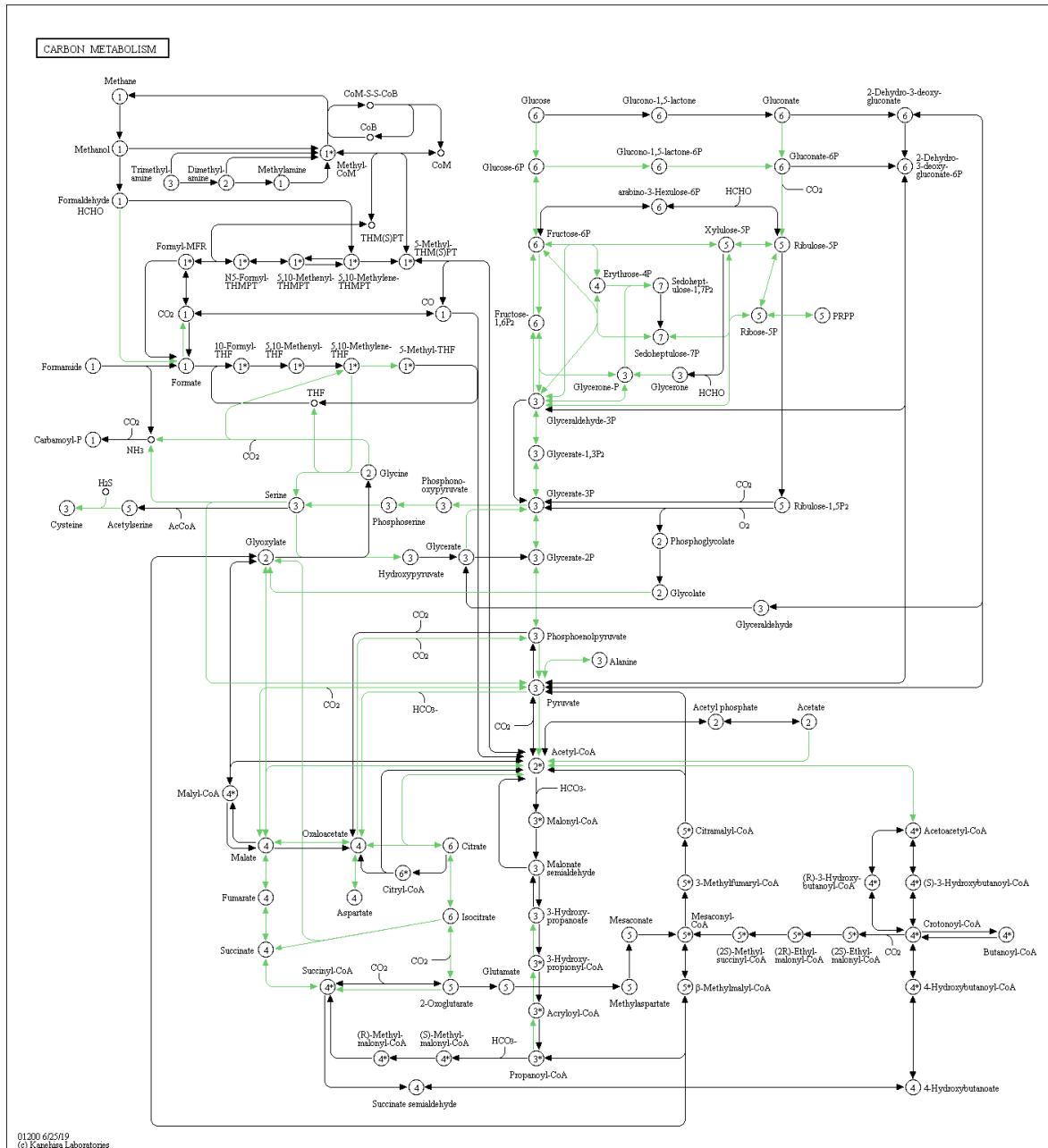


Figure 2.6. Central carbon mechanism of *S. cerevisiae* obtained from KEGG [43].

### 3. MATERIALS AND METHODS

#### 3.1. Consensus *S. cerevisiae* Metabolic Model

Variety of *S. cerevisiae* genome-scale metabolic models have been used since 2003, and each reconstructed model introduced more manual curations, increasing gene numbers from annotations and better predictions regarding the previous ones [47]. A consensus genome-scale metabolic model of *S. cerevisiae*, Yeast8, is presented in an open-source, version-controlled maintainable way in 2019, claiming that the model can be represented and investigated in a systematic way using Git (<https://git-scm.com/>) and GitHub (<https://github.com/>) as a hosting service for the model repository [48]. Systematic way of Yeast8 enables to study simultaneously in collaborative studies, provides record keeping of model changes, version updates, where each version of can be released periodically and accessible all the time (Figure 3.1).

Yeast8 model can be considered as an updated version of Yeast7 [49] with additional corrections based on the annotations available in KEGG and ChEBI, and several gene inclusions from the model iSce926 [50]. Final version of Yeast8 to date, version 8.3.4 released on July 28, has 3991 reactions, 2691 metabolites, 1149 genes and 14 intracellular compartments. Additional statistical analysis on its stoichiometric matrix can be seen in Figure 3.2.

All simulations in the Methods section are done on the model Yeast8 v8.3.4 which is hosted in Github (<https://github.com/SysBioChalmers/yeast-GEM>). All optimization problems were solved with the COBRA Toolbox v3.0.6 in MATLAB (version 9.7.0.1216025 (R2019b)), using Gurobi solver (version 8.1.1).

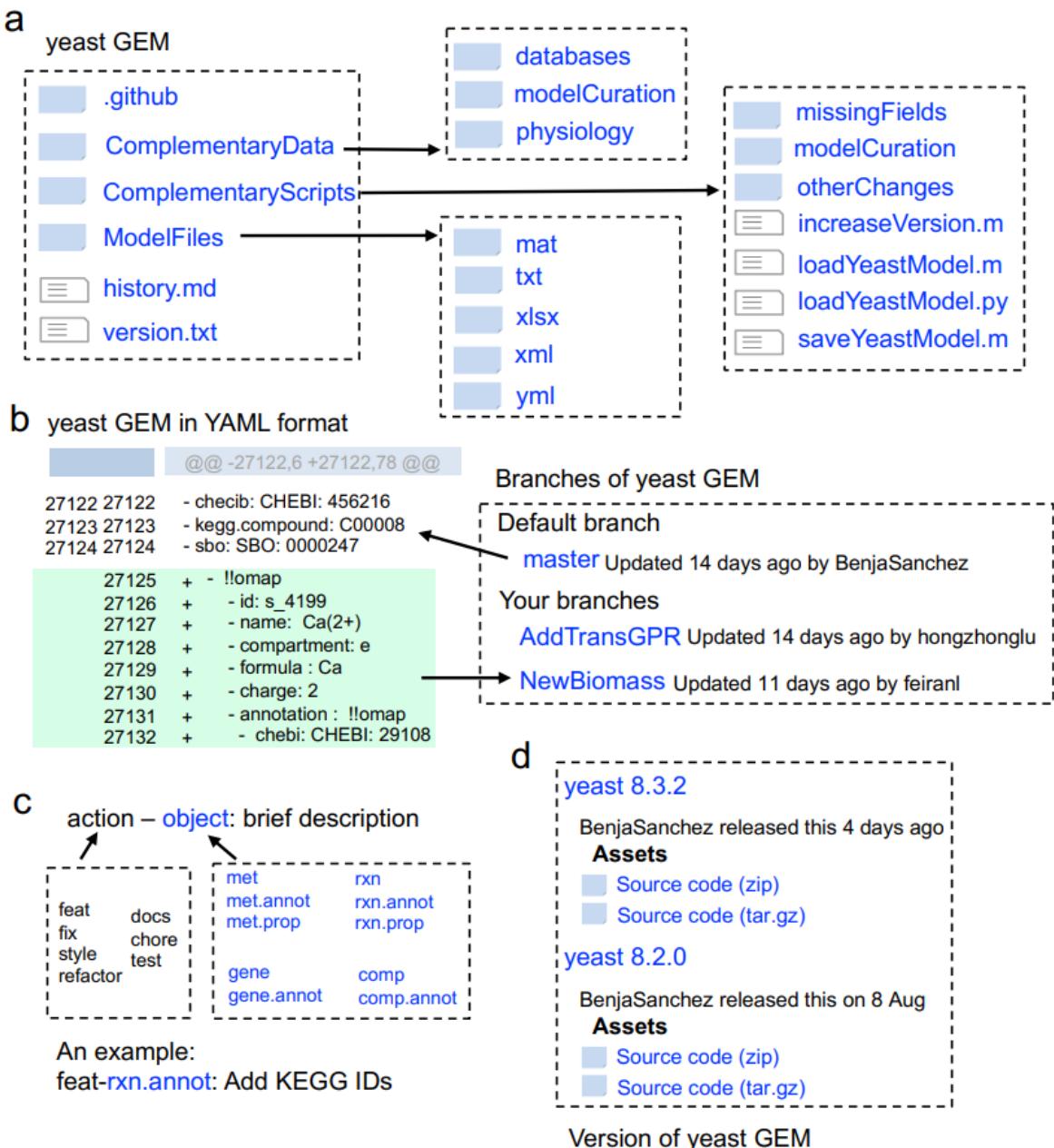


Figure 3.1. Repository of yeast GEM on GitHub. Figure is taken from [48]. will be redrawn in the final version of the thesis.

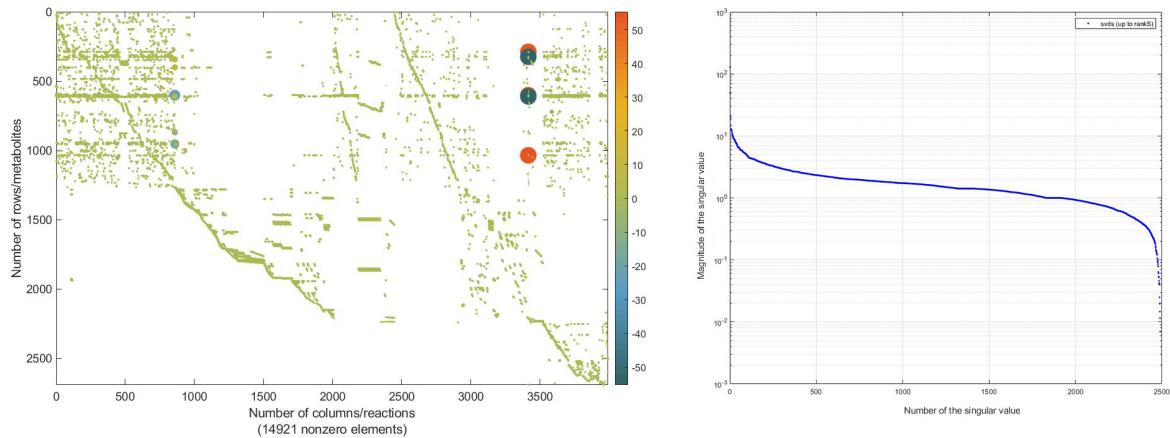


Figure 3.2. Coefficients and singular values of the stoichiometric matrix of Yeast8

### 3.2. Flux Balance Analysis

Flux balance analysis (FBA) assumes that the living cells act as they optimized their lives towards some goal, and as if they were at steady state. To be more clear, steady-state assumption indicates that the metabolites are both produced and consumed at the same rate in a cell, without an accumulation. Therefore, in this system, metabolites are constrained by only the stoichiometric coefficients arised from mass balance of metabolites. FBA solves a set of ordinary differential equations regarding to the stoichiometric matrix:

$$S_{m \times n} \cdot v = 0 \quad (3.1)$$

where  $S$  is the matrix of the stoichiometric reaction coefficients with  $m$  number of metabolites (as rows) and  $n$  number of reactions (as columns), and  $v$  is the vector of all associated reaction fluxes (mmol/gDWh). Because the matrix  $S$  usually has more reactions than metabolites ( $m < n$ ), the system can result multiple solutions, and being called an underdetermined system (read more on Section 2.2.1). To solve it for an optimal solution, additional constraints or an objective function is required.

A "growth reaction" is usually included in the reactions of the system to represent

the "goal" in the definition of living systems. Growth reactions act as the final consumption of metabolites necessary for the biomass production or cell replication. Additional to the growth, several exchange reactions (uptake or secretion of metabolites from or into extracellular space) are also included. Since the concentrations of extracellular metabolites are measurable experimental, constraints can be applied to exchange reaction fluxes to shrink solution space. The more constraints introduced into the system, such as reversibility of reactions or known rate values, result smaller solution space. The growth reaction is usually used as an objective function to determine a unique solution from this solution space. The linear problem appears as:

$$\max_v \quad c^T \cdot v \quad (3.2)$$

$$\text{subject to} \quad S_{m \times n} \cdot v = 0 \quad (3.3)$$

$$v_{lb} \leq v \leq v_{ub} \quad (3.4)$$

where  $c$  is the objective function vector,  $v$  is the vector of fluxes,  $S$  is the stoichiometric matrix as in above equation. Subscripts lb and ub are the lower and upper boundaries on  $v$ . These constraints defines a feasible region of the problem. Coefficients of the biomass constituents are defined as the same as the batch conditions in the reference article [51], for the reason that detailed knowledge is not available in the acquired experimental data (see section 3.3). Coefficients for the final biomass equation can be found in the Table 3.1.

Table 3.1. Biomass coefficients that are used in the FBA simulation (numbers are not updated)

<b>Constituent</b>	<b>Coefficient</b>
Protein	3.703704
RNA	0.37037
DNA	0.018519
Lipid	0.041667
Glycogen	0.030864
Trehalose	0.029214
Mannan	0
Glucan	2.469136
Maintainance	40

### 3.3. Experimental Data Acquisition

Extracellular metabolomics data are obtained from Cakar's Lab [52]. Briefly, they perform ethyl methane sulfonate (EMS) mutagenesis on the prototrophic *Saccharomyces cerevisiae* strain CEN.PK 113-7D (MAT $\alpha$ , MAL2-8c, SUC2) to increase the genetic diversity as an evolutionary engineering selection strategy. Cells were inoculated in 2% Yeast Minimal Media (YMM), and the extracellular concentrations of glucose, ethanol, glycerol and acetate were measured at different time points. OD<sub>600</sub> values were determined by a spectrophotometer. Additionally, cell dry weight analysis was conducted to determine biomass production. Acquired extracellular metabolite concentrations, OD<sub>600</sub> values and dry weights of the reference strain (without mutagenesis) were used in this study are collected in Table 3.3 and Table 3.2.

Table 3.2. Measured OD<sub>600</sub> and cell dry weight values of reference strain.

Time (h)	OD600	ln(OD600)	Cell DW (g/L)
0	0.21	-1.560647748	-
3	0.53	-0.634878272	-
6	1.76	0.565313809	0.9
7.5	2.66	0.978326123	-
9	4.46	1.495148766	1.9
12	5.31	1.669591835	-
15	5.88	1.771556762	-
18	5.83	1.763017	2.32
21	6.07	1.803358605	-
24	5.87	1.769854634	-
30	6.14	1.814824742	2.26
40	6.44	1.86252854	-
46	6.36	1.850028377	-
50	6.3	1.840549633	-
54	6.55	1.87946505	-
63	6.54	1.877937165	-
67	6.88	1.928618652	-
72	6.97	1.941615225	2.66

Table 3.3. Measurements of extracellular concentrations.

Time(h)	Glucose(g/L)	Ethanol(g/L)	Glycerol(g/L)	Acetate(g/L)
0	19.99	0	0	1.08
3	17.98	0.58	0.02	1.24
6	15.85	1.2	0.06	1.16
9	12.21	3.39	0.18	1.37
12	9.18	7.97	0.61	2.45
15	0.4	8.17	0.69	2.46
27	0	8.28	0.76	2.6
46	0	8	0.77	2.45
50	0	6.62	0.64	2.02
54	0	5.74	0.55	1.73
58	0	5.46	0.54	1.74
72	0	3.72	0.49	1.33

As the slope in the curve of  $\ln OD_{600}$  as a function of time gives the growth rates of cells, natural logarithm of  $OD_{600}$  values were calculated to obtain specific growth rates by using the equation 3.5.

$$\mu = \frac{\Delta \ln OD_{600}}{\Delta t} \quad (3.5)$$

In order to determine uptake and secretion rates of the metabolites, the steady-state assumption is applied in three hours intervals as the shortest measured time-points. Missing data on cell dry weights are estimated from the  $OD_{600}$  values, and these cell dry weight data is used to calculate fluxes (in the unit of mmol/gDWh). Measurement of the cell dry weight at the 3rd hour was crucial for the steady-state assumption, however data was not available from the experiments. Curve trend of the  $OD_{600}$  plot is used as a guide to estimate cell dry weight (Figure 3.3).

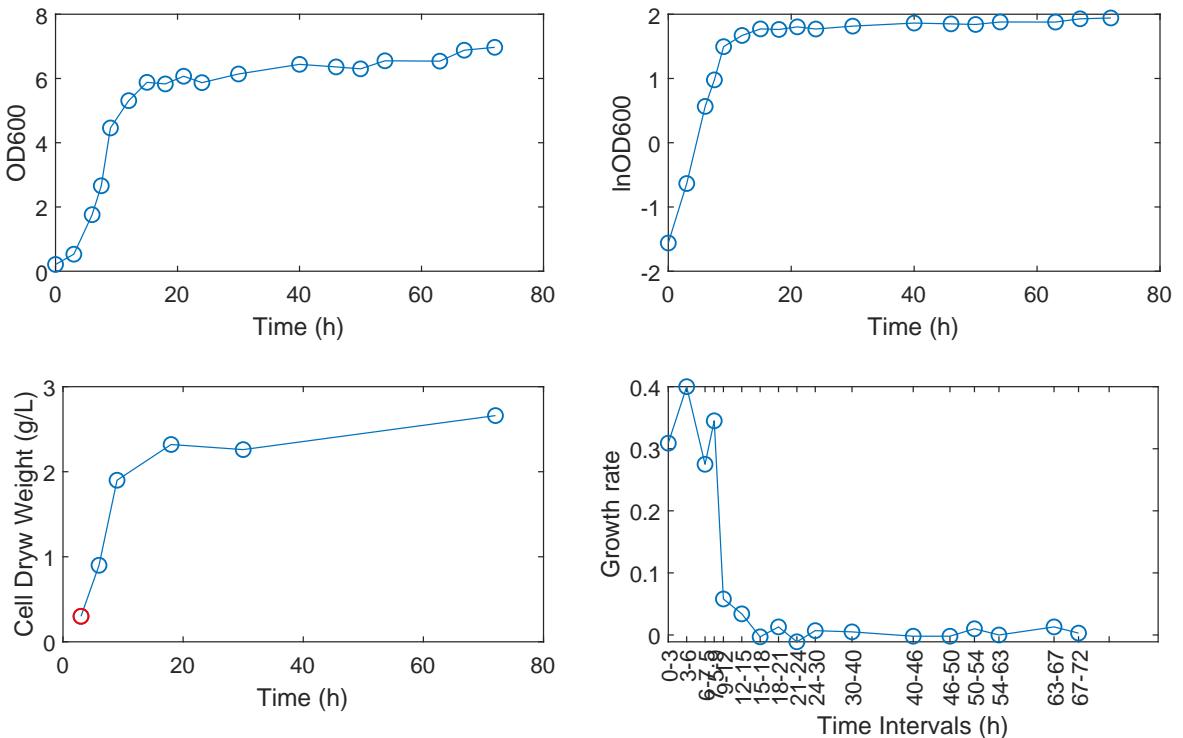


Figure 3.3. OD<sub>600</sub>, lnOD<sub>600</sub>, cell dry weights and growth rates graphs. Estimated missing cell dry weight data is shown in red color.

### 3.4. Batch Simulation

In order to simulate batch conditions where minimal yeast medium is used, all the exchange reactions in the model are blocked first (lower bounds are set to 0). Then, only the exchange reactions of ions that are available to the cells in the experimental design (ammonium, phosphate, sulphate, iron(2+), H+, water, chloride, Mn<sup>2+</sup>, Zn<sup>2+</sup>, Mg<sup>2+</sup>, sodium, Cu<sup>2+</sup>, Ca<sup>2+</sup>, potassium) are set free (lower bounds are set to -1000), means that cells can uptake as it needs. While oxygen and glucose uptake rates decreased from 20 mmol gDWh<sup>-1</sup> and increased to 20 mmol gDWh<sup>-1</sup>, respectively, fluxes of ethanol, acetate, glycerol, formate, succinate secretion reactions with the growth rate is collected.

### 3.5. Integration of Enzymatic Constraints

Solution space of the Yeast8 can be further constrained by limiting fluxes of reactions with integration of enzymatic informations by using GECKO (GEM with Enzymatic Constraints using Kinetic and Omics data) method [53]. In GECKO methodology, if a reaction has an annotated enzyme requirement in order to occur in the cell, extra enzyme entity is included in the reaction equation itself. Stoichiometric coefficient of this entity is defined by its kinetic information and abundance in the cell (Figure 3.4). S matrix of the system is expanded with the addition of new rows for the enzymes and new columns for the each enzyme's usage.

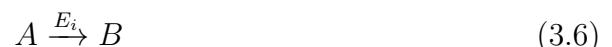
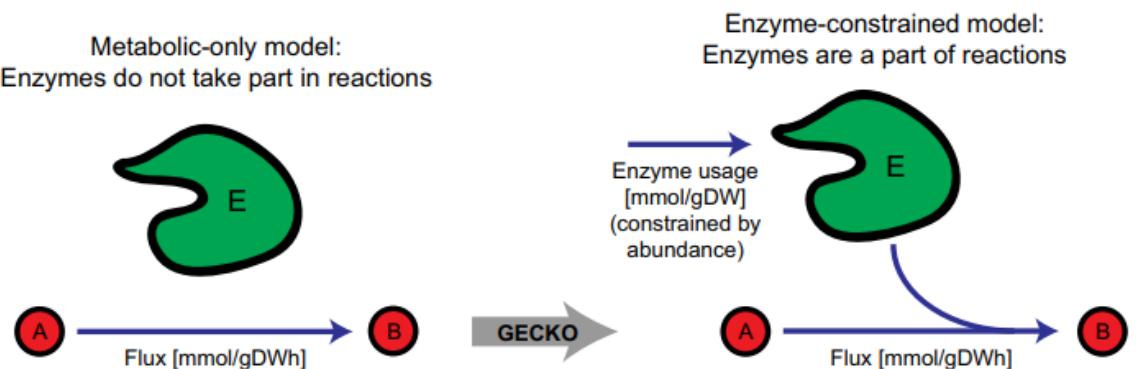


Figure 3.4. GECKO, will be redrawn.

The idea behind this methodology arises from the biochemical constraints, such that the maximum rate of a reaction is smaller or equal to its enzyme's turnover number and the concentration (mmol/gDW).

$$v_j \leq k_{cat}^{ij} \cdot [E_i] \quad (3.8)$$

Enzymes in the reaction equations must be considered as pseudo-metabolites because they do not affect the mass balance of the reaction, i.e. enzymes are not consumed

in the reactions but they are occupied for a short period of time. To maintain mass balances of enzymes, an overall enzyme usage pseudo-reaction is introduced into system in order to supplement enzymes into reactions, similar to exchange reactions:



If the flux carried by this reaction is  $e_i$ , we can say that  $e_i$  ranges from 0 (i.e. no enzyme available) to a maximum value of  $[E_i]$  (i.e. all the enzyme is used in the reaction):

$$0 \leq e_i \leq [E_i] \quad (3.10)$$

Then a mass balance for the enzyme  $E_i$  can be defined under steady state assumption:

$$-n_{ij} \cdot v_j + e_i = 0 \quad (3.11)$$

By rearranging the equations 3.10 and 3.11, we obtain:

$$v_j \leq \frac{1}{n_{ij}} \cdot [E_i] \quad (3.12)$$

and if we compare this equation with the equation 3.8, we can finally calculate the stoichiometric coefficient for the enzyme.

$$n_{ij} = \frac{1}{k_{cat}^{ij}} \quad (3.13)$$

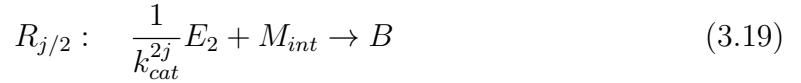
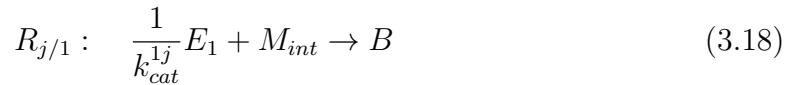
However the equation 3.8 does not hold in all cases since reactions can be more complex. For isozymes in which multiple enzymes catalyze the same reaction, the equation becomes:

$$v_j \leq \sum_i k_{cat}^{ij} \cdot [E_i] \quad (3.14)$$

Since the reaction can be catalyzed by all the isozymes equally, new reactions can be defined for each isozyme (note that each enzyme can have different  $k_{cat}$  values). For example, if we think that the equation 3.6 had two isozymes,  $A \xrightarrow{E_1 \text{ or } E_2} B$ , the new reactions defined would be,



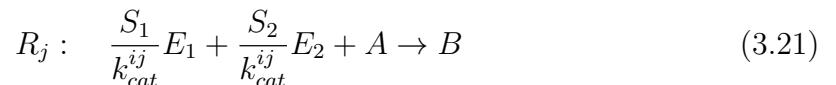
These additional reactions disrupt the boundaries for the initial reaction. In order to maintain original boundaries, another "arm-reaction" is also introduced to the system where it acts as an intermediate metabolite.



For an enzyme complex catalyzing a single reaction, since the reaction is catalyzed by all the enzymes which share the same  $k_{cat}$  value, the equation 3.8 becomes:

$$v_j \leq k_{cat}^{ij} \cdot \min_k \frac{[U_{ik}]}{S_{ik}} \quad (3.20)$$

where  $[U_{ik}]$  is the concentration of the subunit of the catalyzing enzyme  $E_i$ , and  $S_{ik}$  is the stoichiometry of the subunit. For the reaction, if we think that the equation 3.6 was catalyzed by an enzyme complex of 2 subunits,  $A \xrightarrow{E_1 \text{ and } E_2} B$ , then it would be:



Finally, for the reversible reactions, two reactions must be defined for both forward

and backward direction reactions with the same catalyzing enzyme but possibly with different  $k_{cat}$  values. Assume the equation 3.6 is reversible, following equations would be introduced to the system:



### 3.6. Integration of Transcriptome Data

Transcriptome data is initially converted into protein abundances and used to change the stoichiometry of the enzyme usage pseudo-reaction. Will be explained when a solid function for expression=abundance assumption is found.

### 3.7. Phenotype Phase Plane Construction

As previously mentioned, there is no single solution to the linear problem of the model. Phenotype phase planes (PhPP) are used to describe all feasible metabolic states in a two or three dimensional surfaces, depending on the number of metabolites chosen to see how they affect the objective function [54]. In general, for aerobic models, various levels of glucose and oxygen availability through their uptake reactions are used to generate PhPP surfaces in three dimension with objective function. Fundamentally, PhPP construction refers to a double robustness analysis on the model for selected reactions.

### 3.8. Flux Variability Analysis

Flux variability analysis (FVA) finds the minimum and maximum available fluxes for each reaction while obeying the provided constraints (for example fixed glucose uptake or growth rate). FVA is mainly used to evaluate the robustness of the model [55], to find alternative optimum states [56], to check flux distributions when growth is not

at optimum level [57], and it has many other applications [58].

FVA, similar to FBA, solves two optimization problems for each reaction:

$$\max_v / \min_v \quad v_i \quad (3.24)$$

$$\text{subject to} \quad S_{m \times n} \cdot v = 0 \quad (3.25)$$

$$w^T \cdot v \geq \gamma \cdot Z_0 \quad (3.26)$$

$$v_{lb} \leq v \leq v_{ub} \quad (3.27)$$

where  $w$  is the objective function equals to  $c$  in the problem 3.3,  $Z_0 = w^T \cdot v_0$  describes an optimal solution to the problem 3.3,  $\gamma$  is an indicator to check whether the FVA is done at the optimal state (where objective flux is the same and  $\gamma = 1$ ) or any other state (where  $0 \leq \gamma < 1$ ).

### 3.9. Random Sampling of Solution Space

Constraints applied to a model define a solution space, a convex polytope, where every flux distribution is accessible. Random sampling of the solution space is an unbiased tool to explore metabolic models. Mainly, Markov Chain Monte Carlo methods are used to sample this space using algorithms such as (Artificially Centered) Hit-and-Run (HRB) [59, 60] algorithm, and this method has proven to be helpful in the analysis of genome-scale metabolic models [61]. Briefly, the random sampling method collects points that are uniformly distributed in the solution space and calculates the most probable flux value for each reaction.

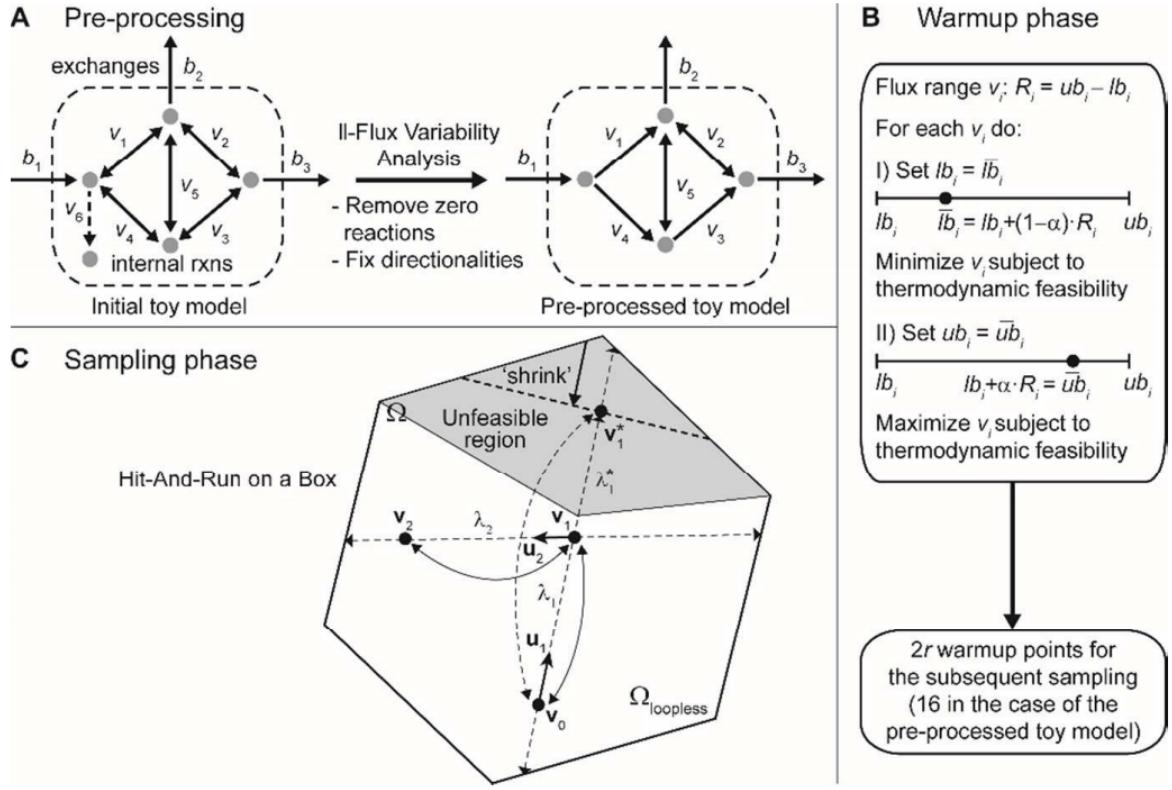


Figure 3.5. Workflow of the ll-ACHRB sampling on a toy model. A) Pre-processing phase, application of loopless-FBA to remove blocked reactions and constraining the directionalities of others. B) Warmup phase, modifying the reaction bounds to more interior space. C) Sampling phase with HRB algorithm. Figure is taken from [60]

Since the computational burden of loopless sampling is high, generated random points in the solution space of Yeast8 includes thermodynamically unfeasible states. Maximum glucose uptake rate was constrained to 1 mmol gDWh<sup>-1</sup> and total of 5000 points are generated with maximum of 120 seconds allotted for the sampling.

## 4. RESULTS \*NOT UPDATED\*

Results section is not updated after GECKO integration! Since the method changes frequently, below figures and texts are not updated and they only give the idea about what is going to be in the final thesis.

### 4.1. Chemostat Simulation: GAM Fitting

In order to make sure the *in-silico* obtained growth rate predictions are in agreement with the physiological kinetic parameters obtained from real experiments, fine adjustment on the energy reactions is a requirement. Since the growth-associated maintenance (GAM) and non-growth associated maintenance (NGAM) energy reactions play a determinant role in simulations, fluxes through these reactions must be constrained to a fixed value. Flux of NGAM is constrained to 0.7 mmol gDWh<sup>-1</sup> for aerobic, and 0 mmol gDWh<sup>-1</sup> for anaerobic simulations as calculated in the previous studies [51]. For the estimation of GAM, since it depends on the biomass composition, findings of a chemostat experiment [62] is used as a guide to fit predictions to. Model is simulated iteratively with a range of values for GAM, and the best fit is found at the level of 55.25 mmol gDWh<sup>-1</sup> (Figure 4.1), GAM flux is constrained accordingly.

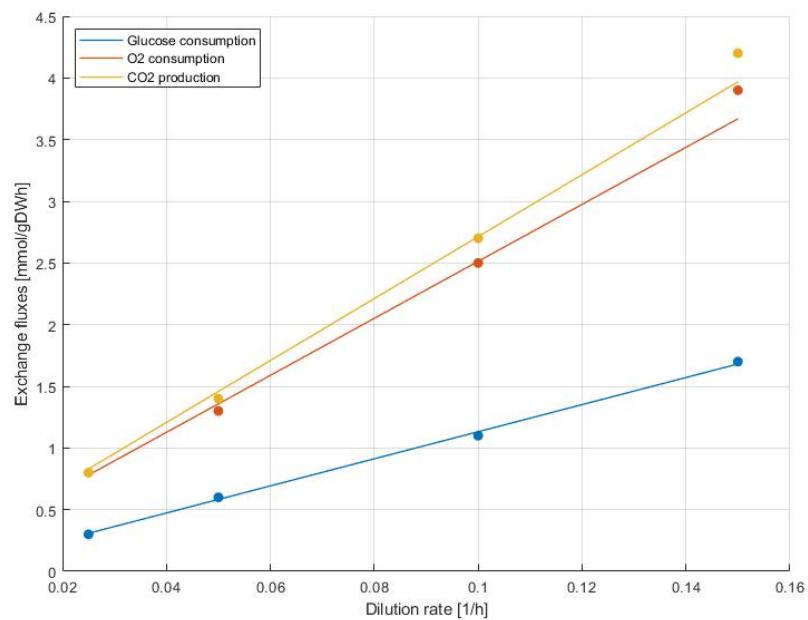


Figure 4.1. Chemostat simulation to re-fit growth associated maintenance.

#### 4.2. Flux Balance Analysis Results

Experimental fluxes... Compared to simulations...

Table 4.1. Calculated flux values.

Time	Metabolite fluxes in mmol/gDWh				Growth h-1
	Glucose	Ethanol	Glycerol	Acetate	
0-3	-12.3963884	13.98837518	0.24131	2.960496	0.30859
3-6	-4.378823762	4.984363569	0.160873	-0.49342	0.400064

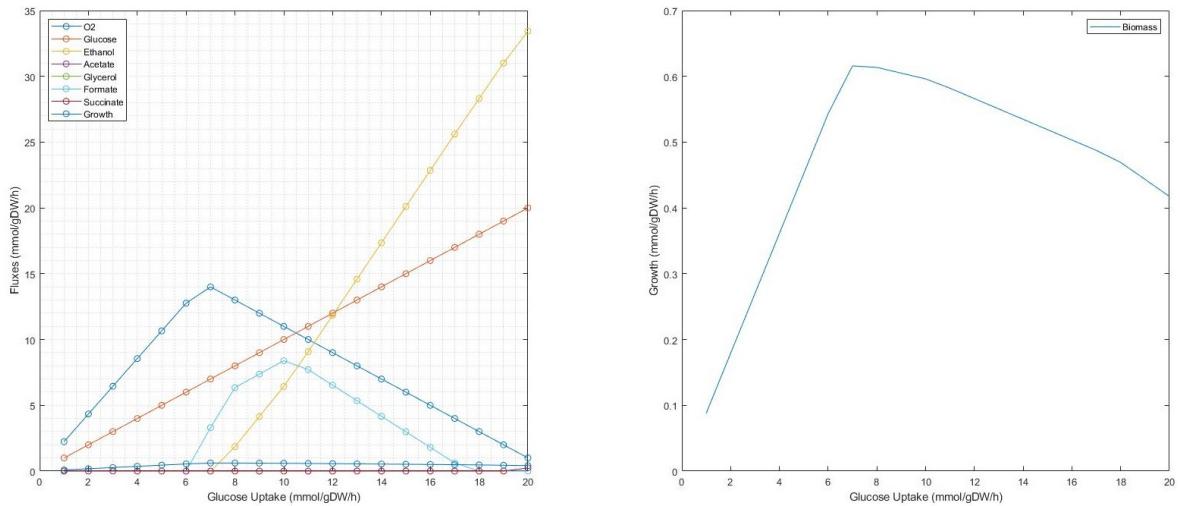


Figure 4.2. Flux balance simulation results where oxygen uptake rate decreased and glucose uptake rate is increased simultaneously. Flux rates of several metabolites on the left, predicted growth rate on the right.

### 4.3. Flux Variability Analysis

Flux variabilities of Yeast8 reactions are analyzed by solving the linear problem with the objective functions to minimize and maximize all reactions iteratively with tolerance value of 1e-9 using the GUROBI solver. Minimum and maximum available fluxes are collected in the iterative process for each reaction, and results are plotted for glycolysis, pentose phosphate pathway and TCA pathway reactions as error boxes (Figure 4.3). Flux values obtained through the ordinary FBA solution are also shown as a line.

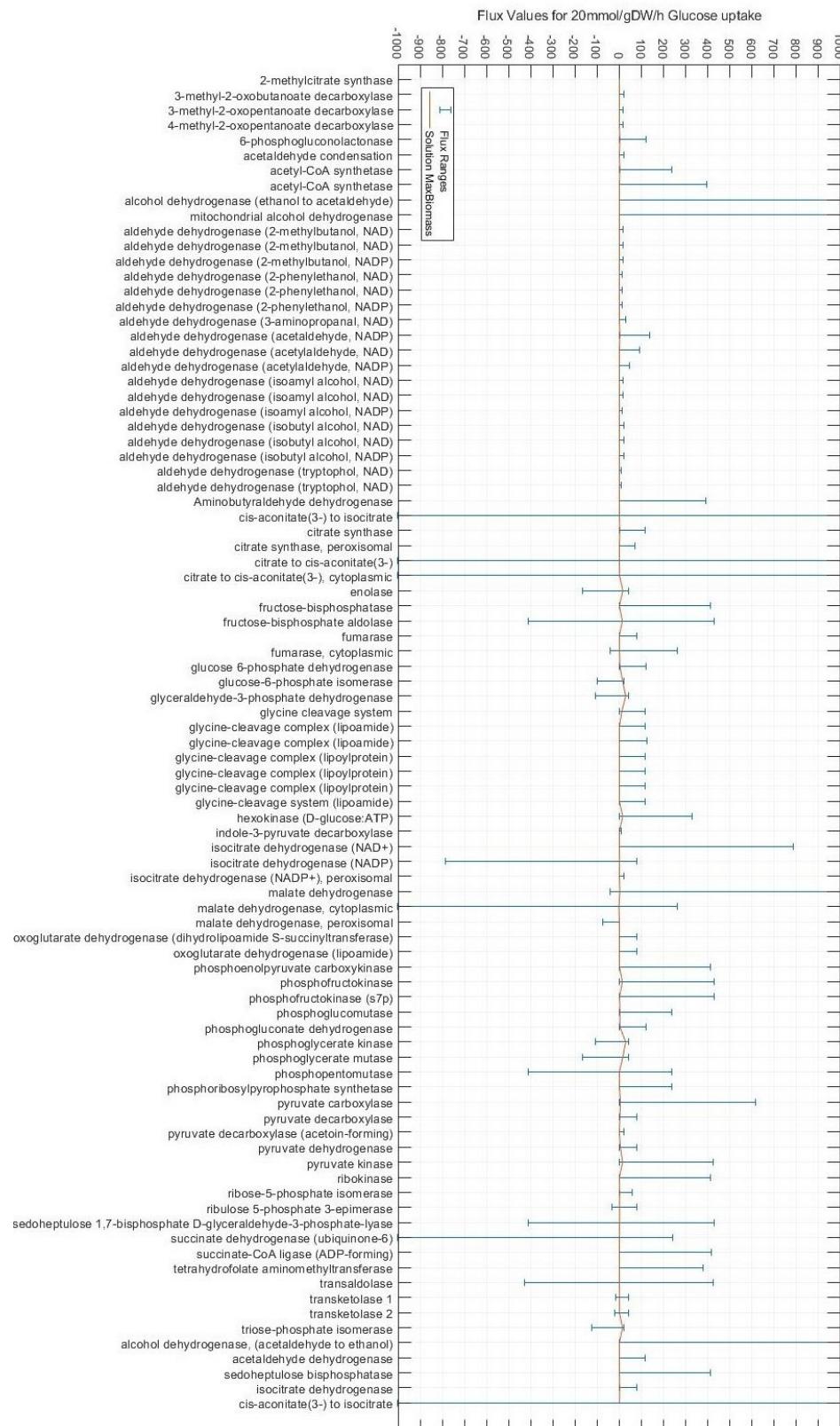


Figure 4.3. Minimum and maximum fluxes of Glycolysis, PPP and TCA reactions.

#### 4.4. Phenotype Phase Plane

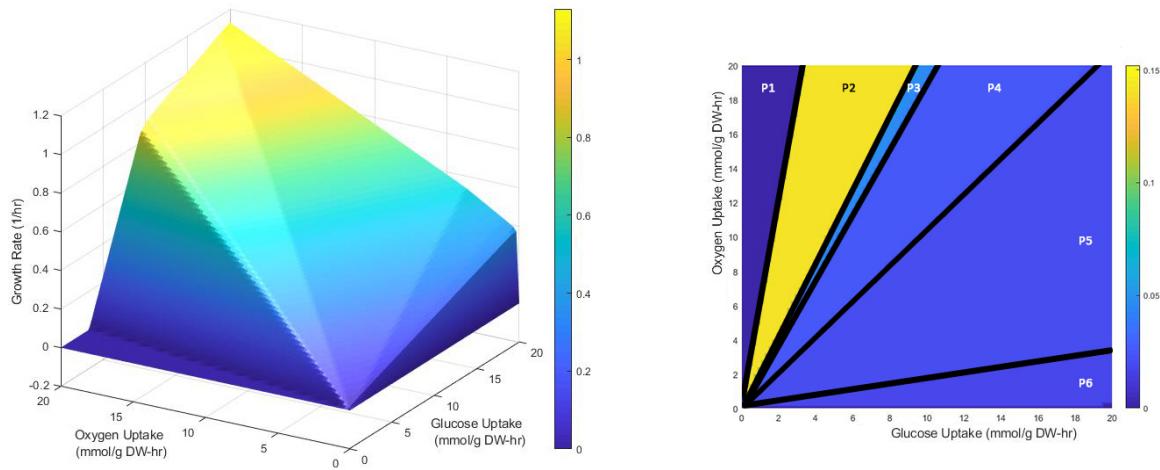


Figure 4.4. Phenotype Phase Plane of Yeast8, corresponding to glucose and oxygen availabilities on the left. Shadow prices of glucose on the right.

#### 4.5. Sampling Results

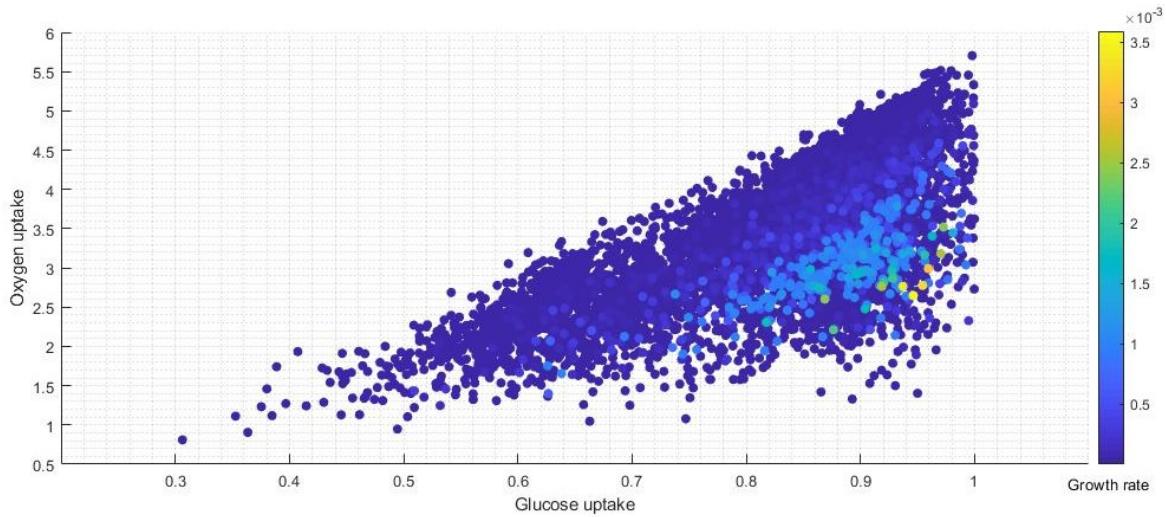


Figure 4.5. Scatter plot of glucose and oxygen uptake points from sampling regarding growth rate.

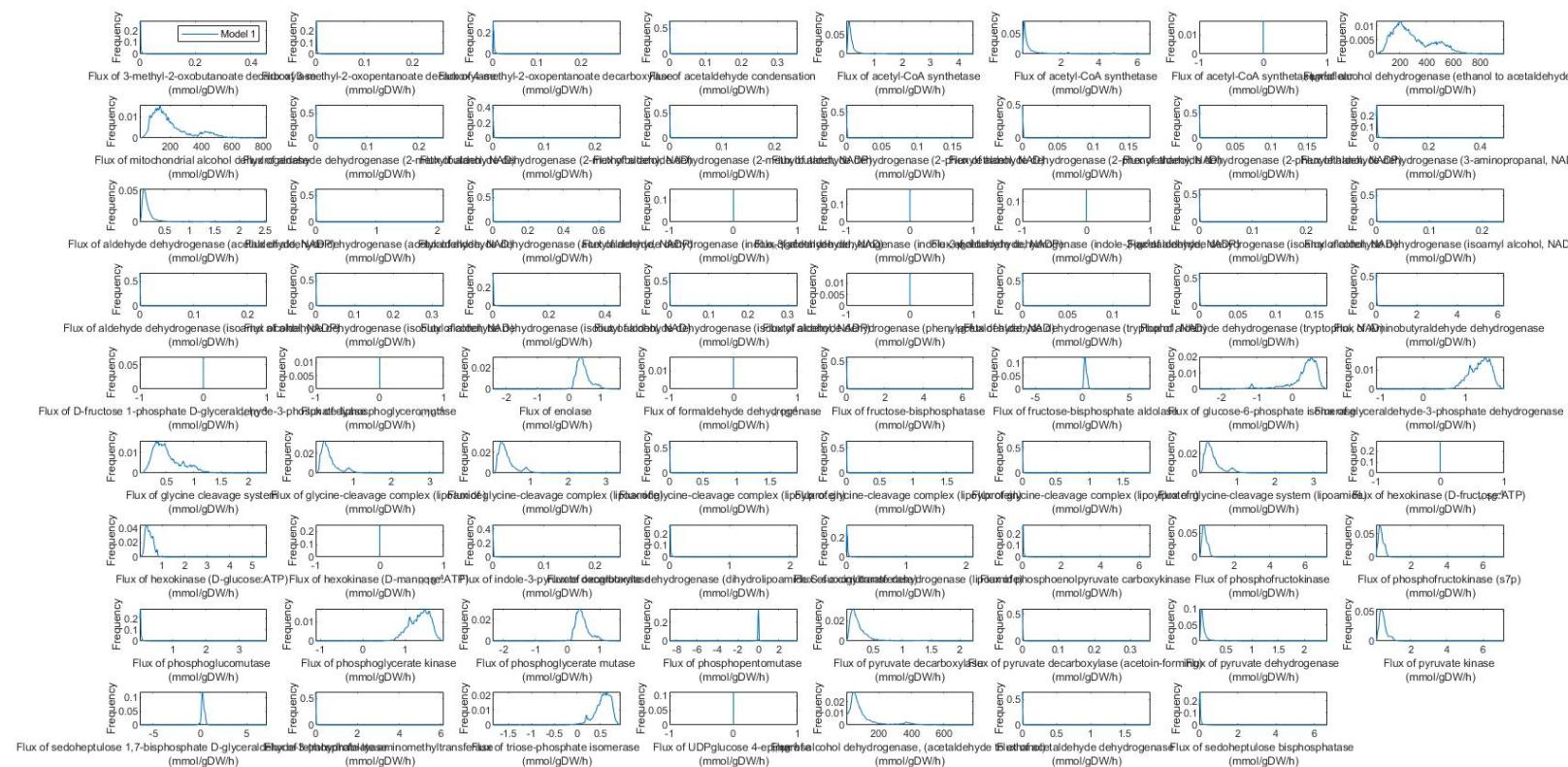


Figure 4.6. Histogram plot of sampled points in glycolysis reactions.

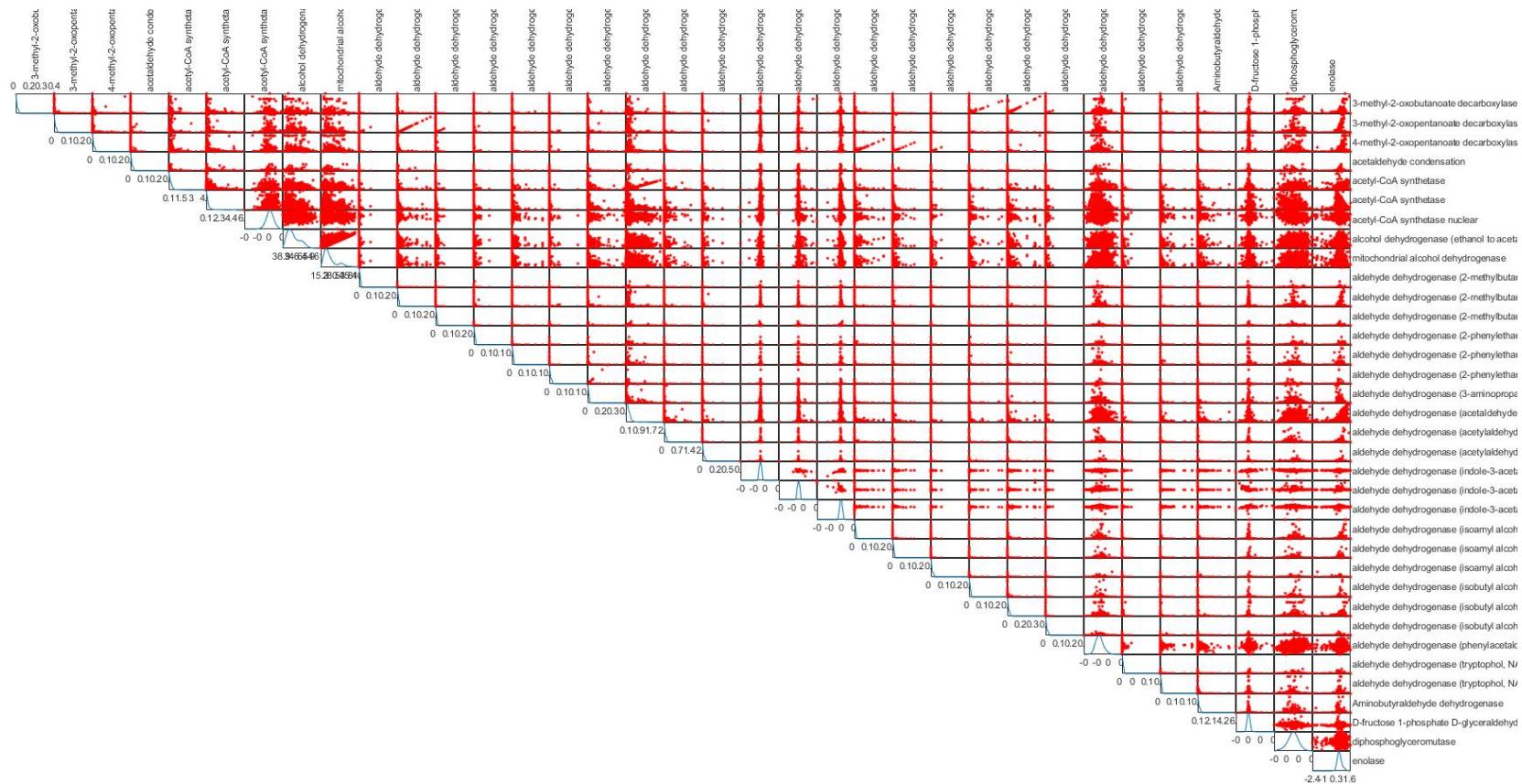


Figure 4.7. Correlations in between glycolysis reactions.

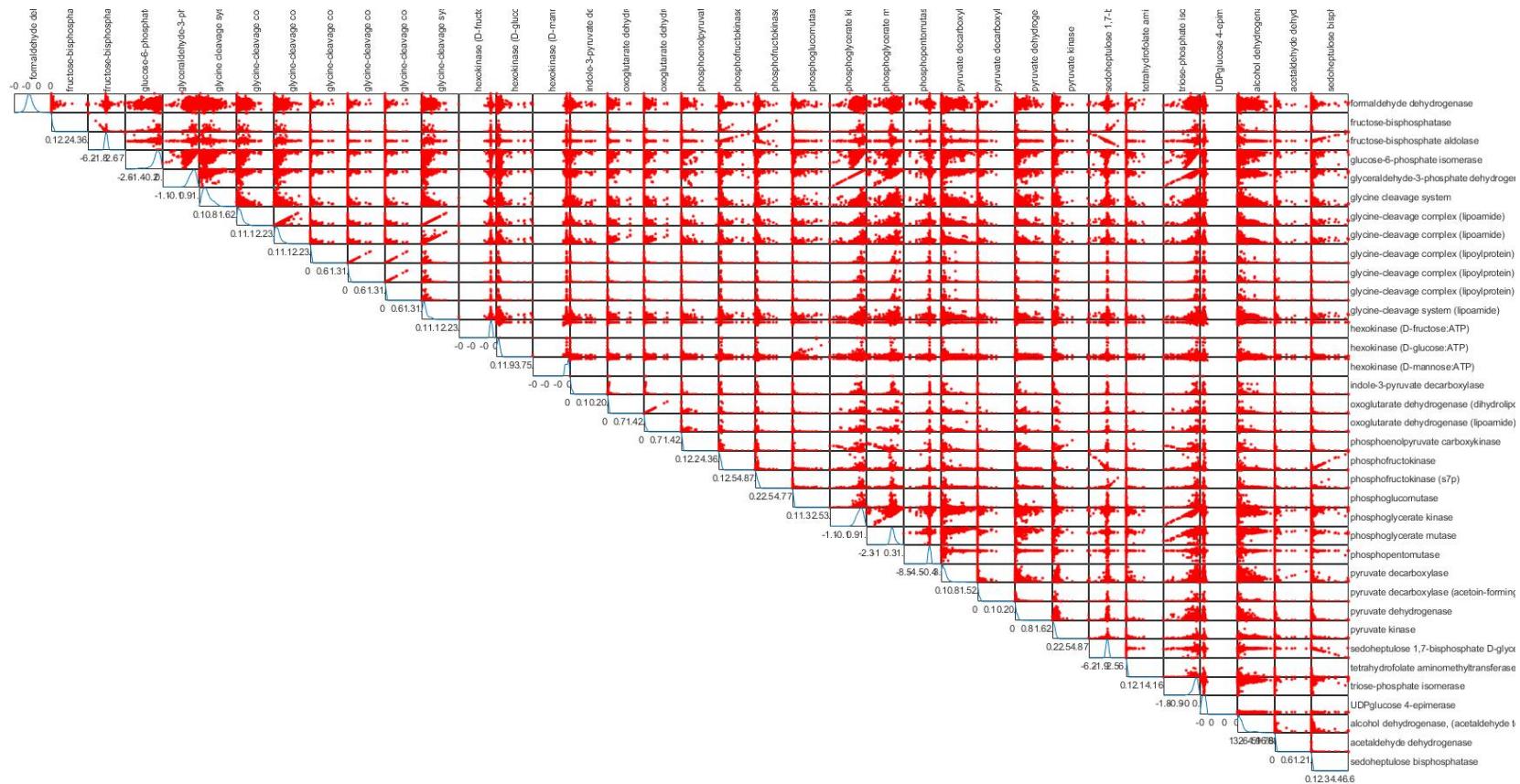


Figure 4.8. Correlations in between glycolysis reactions, continued.

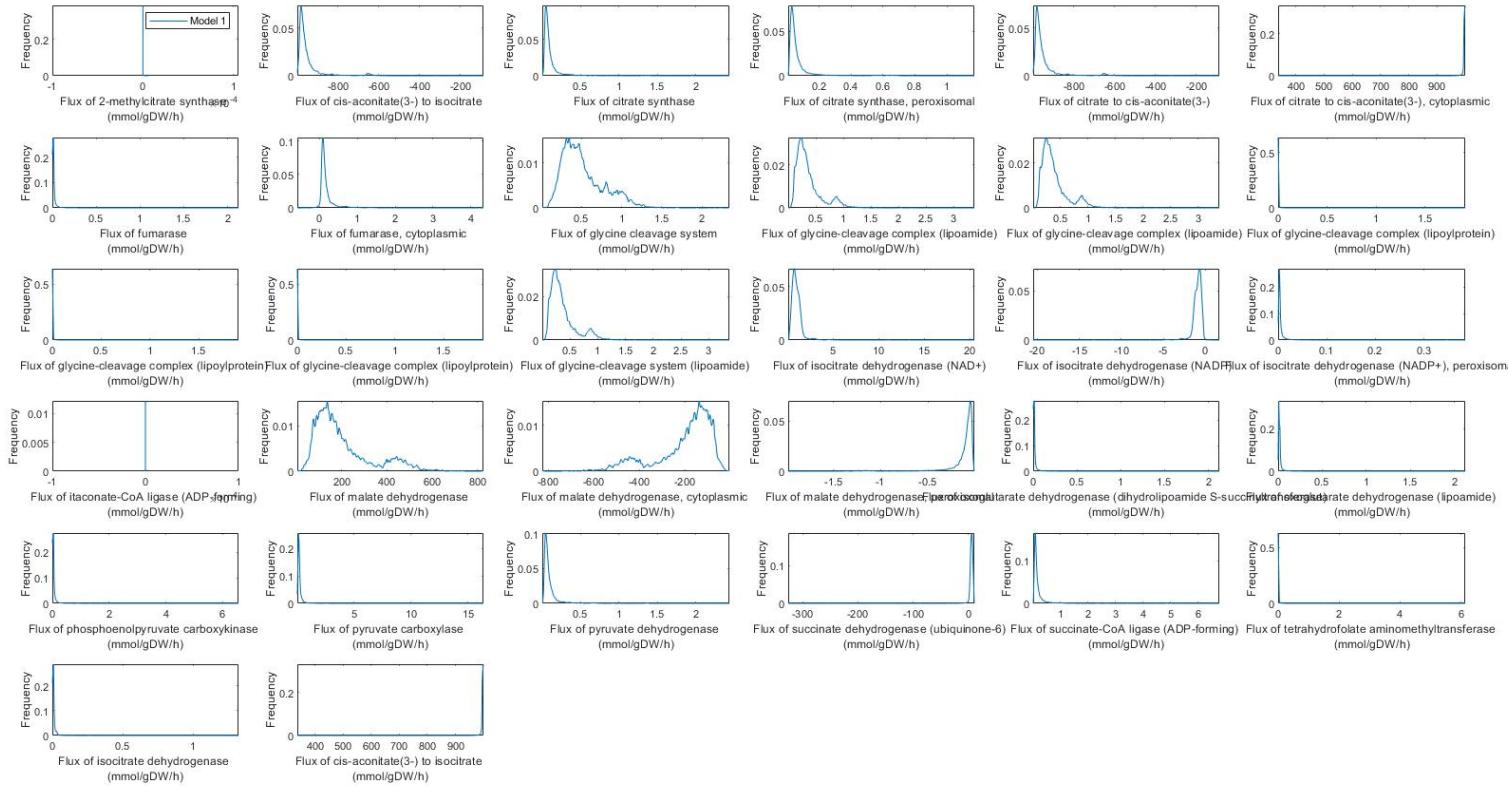


Figure 4.9. Histogram plot of sampled points in TCA reactions.

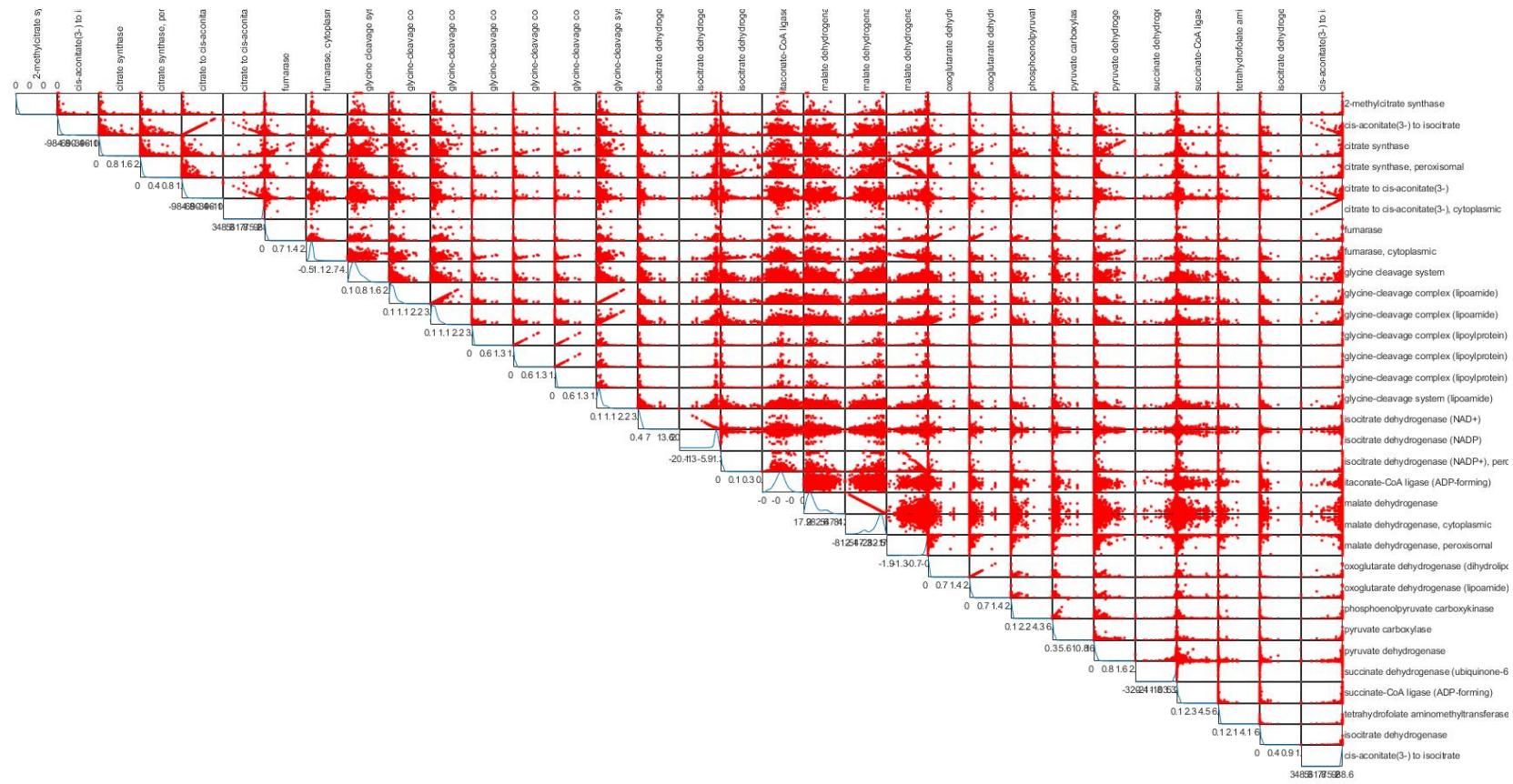


Figure 4.10. Correlations in between TCA reactions

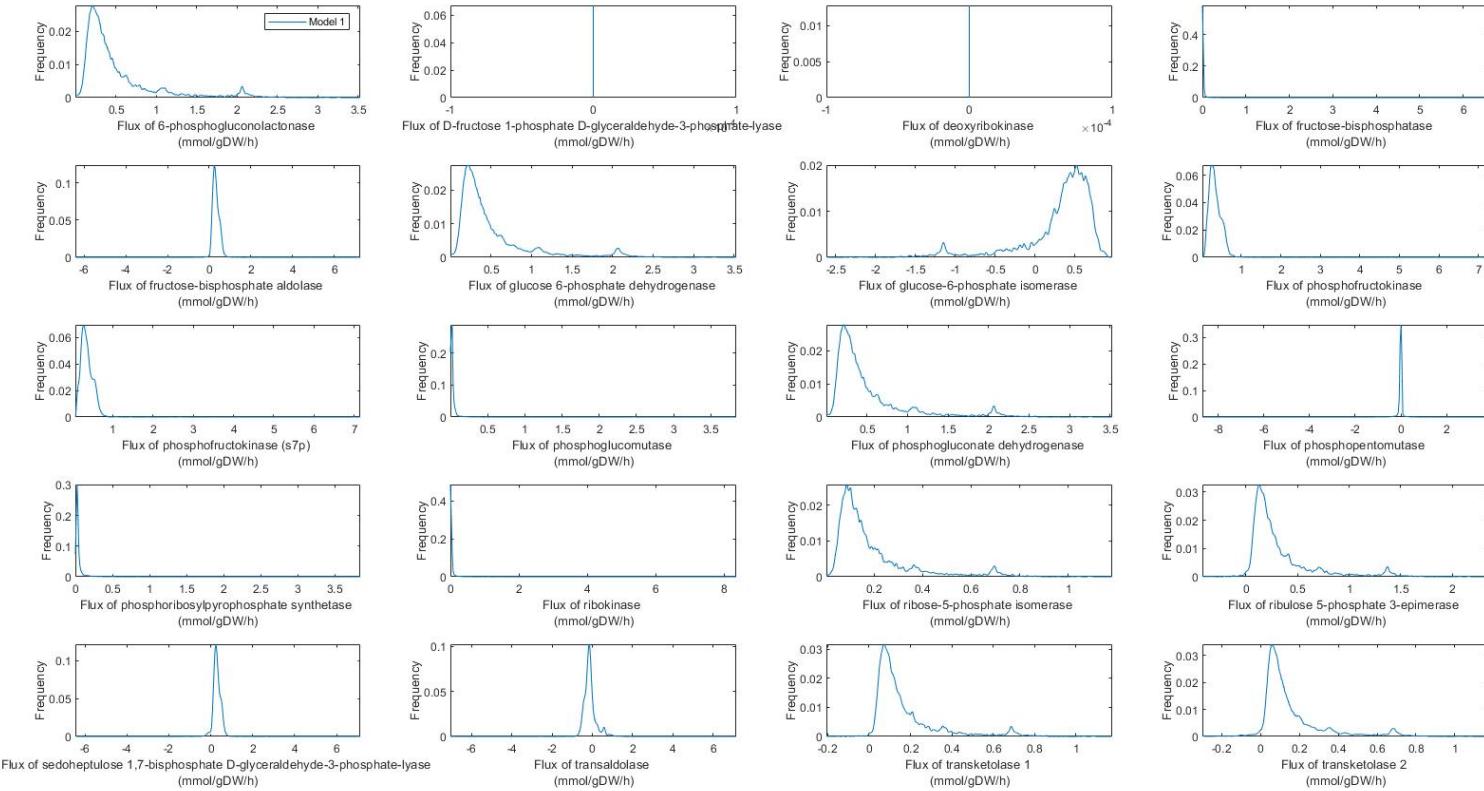


Figure 4.11. Histogram plot of sampled points in PPP reactions.

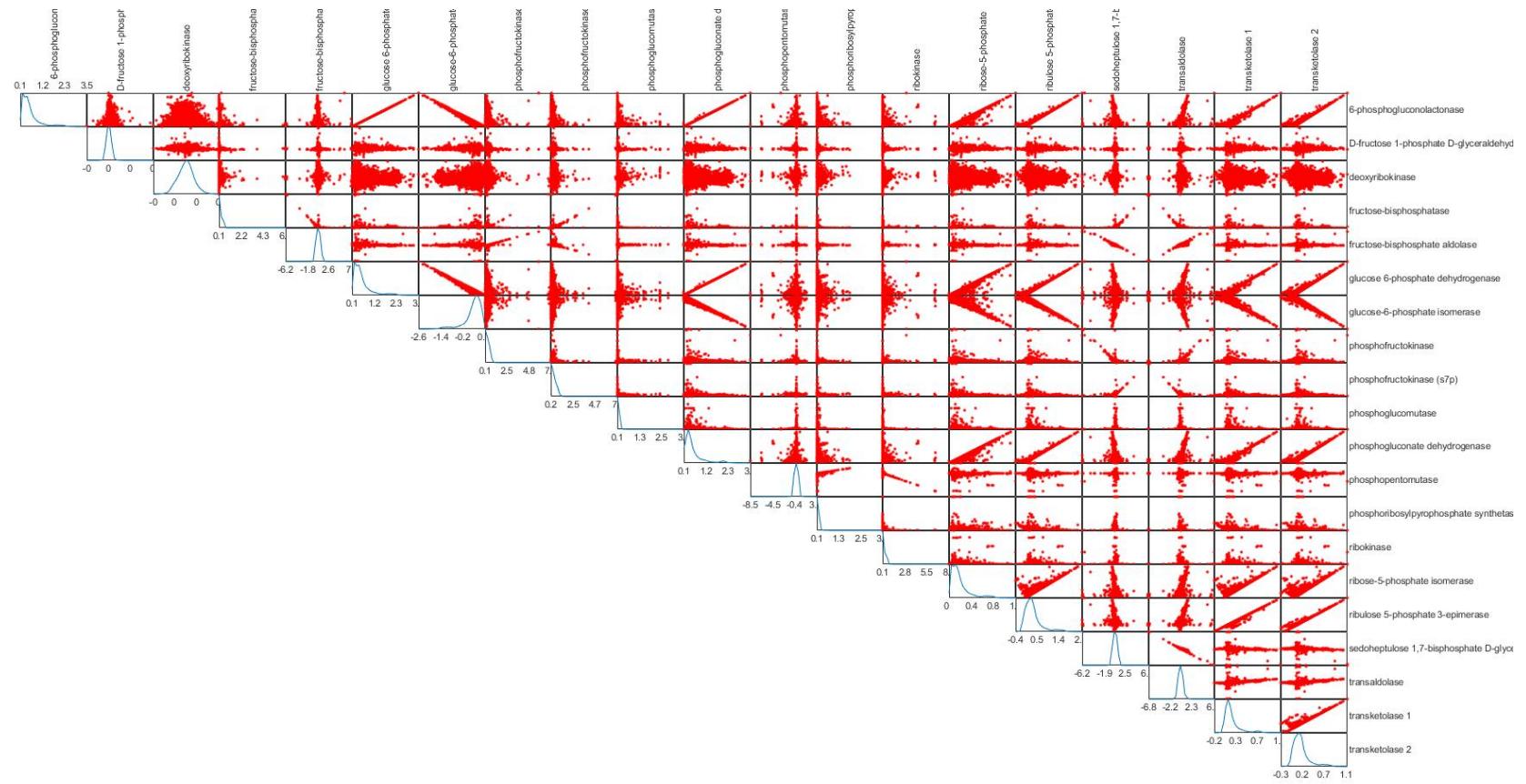


Figure 4.12. Correlations in between PPP reactions

#### 4.6. Single Gene and Reaction Knock-Out Simulations

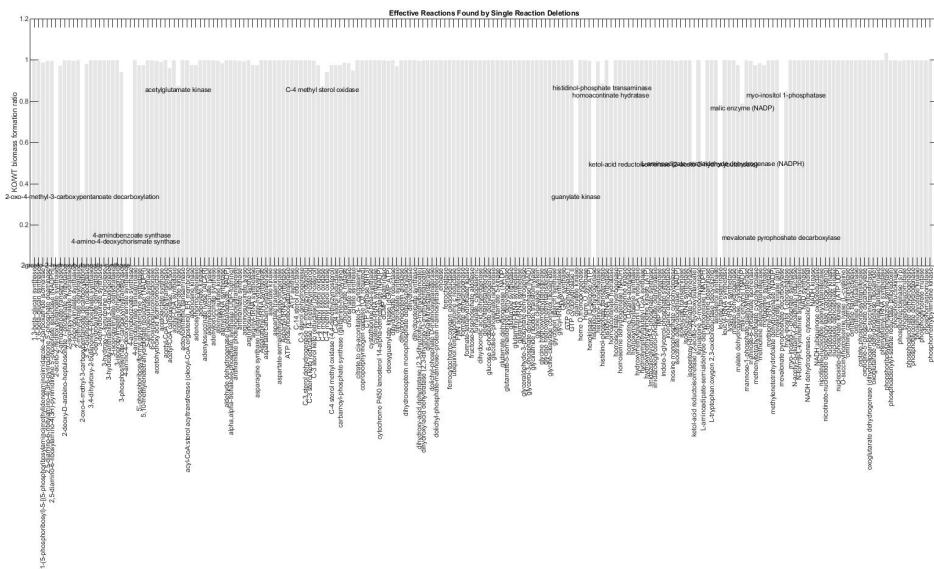


Figure 4.13. Effective reactions (not entirely essential, but their absence changes the flux distribution or value of the optimization function) obtained from single reaction deletion simulations using FBA and their effect on growth rate is plotted.

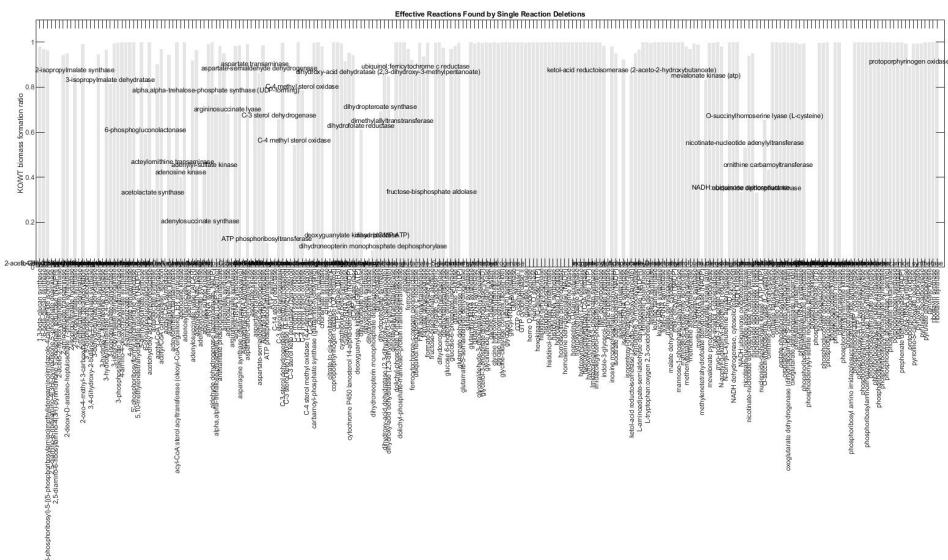


Figure 4.14. Effective reactions (not entirely essential, but their absence changes the flux distribution or value of the optimization function) obtained from single reaction deletion simulations using MOMA and their effect on growth rate is plotted.

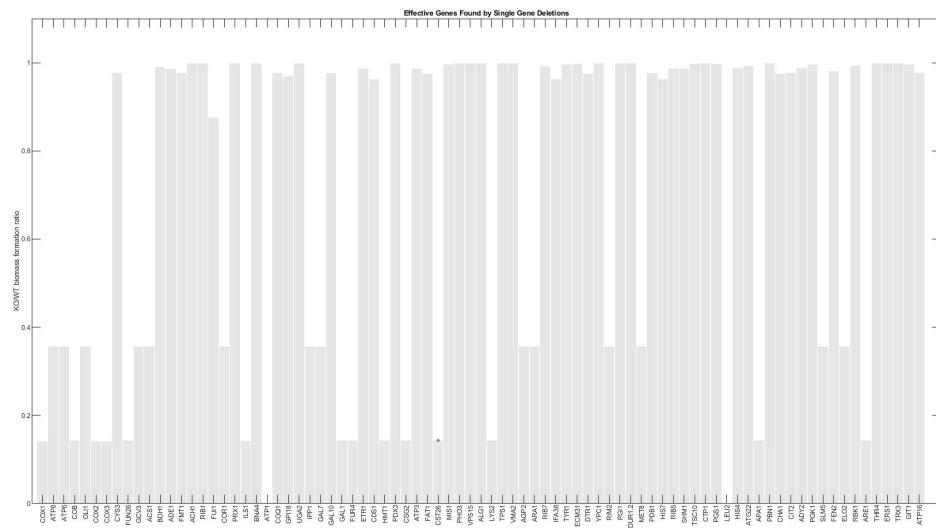


Figure 4.15. Effective genes (not entirely essential, but their absence changes the flux distribution or value of the optimization function) obtained from single gene deletion simulations using FBA and their effect on growth rate is plotted.

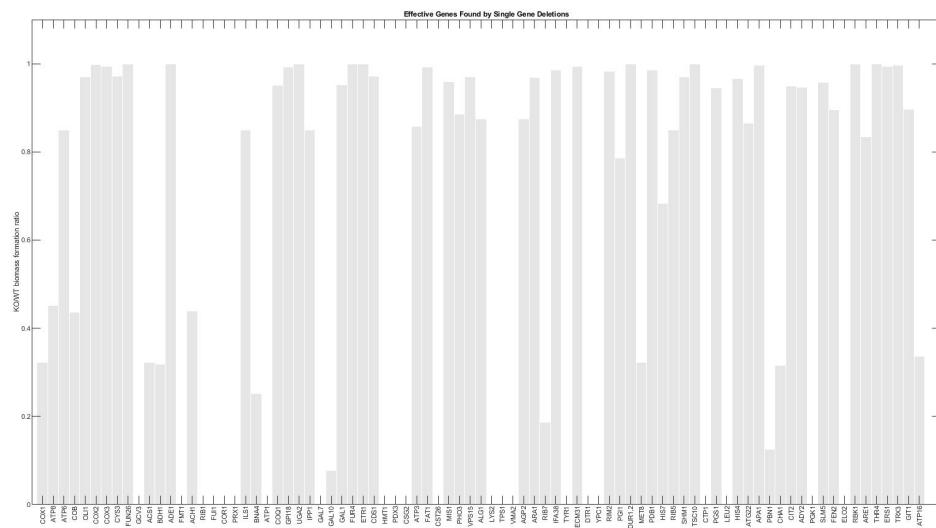


Figure 4.16. Effective genes (not entirely essential, but their absence changes the flux distribution or value of the optimization function) obtained from single gene deletion simulations using MOMA and their effect on growth rate is plotted.

Explainations about knock-out strains, gene descriptions, comparison of found essential genes and their reactions corresponding to essential reactions found will be added.

Table 4.2: Total of 166 genes are found essential, meaning that their absence is crucial to model, without flux going through these genes' reactions system will not be able to grow.

Gene	Name	Name Description	Subsystem
YGL256W, YBR145W	ADH4, ADH5	alcohol dehydrogenase, (acetaldehyde to ethanol), aldehyde, dehydrogenase (2-methylbutanol, 2-phenylethanol, isoamyl alcohol, isobutyl alcohol, tryptophol, 2-methylbutanol, 2-phenylethanol, isoamyl alcohol, isobutyl alcohol, tryptophol, NAD)	Gluconeogenesis, Glycolysis, Fatty acid degradation, Tyrosine metabolism, Biosynthesis of secondary metabolites, Biosynthesis of antibiotics
YER152C	YER152C	2-amino adipate transaminase	Ubiquinone and other terpenoid-quinone biosynthesis, Cysteine and methionine metabolism, Lysine biosynthesis, Tyrosine metabolism, Phenylalanine metabolism, Tryptophan metabolism, Phenylalanine, tyrosine and tryptophan biosynthesis, Biosynthesis of secondary metabolites, Biosynthesis of antibiotics, 2-Oxocarboxylic acid metabolism, Biosynthesis of amino acids,
YDR127W, YDR035W, YBR249C YDR234W	ARO1, ARO3, ARO4 LYS4	2-deoxy-D-arabino-heptulosonate 7-phosphate synthetase, 3-deoxy-D-arabino-heptulosonate 7-phosphate synthetase 2-methylcitrate dehydratase, homoacetinate hydratase	Phenylalanine, tyrosine and tryptophan biosynthesis, Biosynthesis of secondary metabolites, Biosynthesis of antibiotics, Biosynthesis of amino acids
YDR487C YLR044C	RIB3 PDC1	3,4-dihydroxy-2-butanone-4-phosphate synthase 3-methyl-2-oxobutanoate decarboxylase, 3-methyl-2-oxopentanoate decarboxylase, acetaldehyde condensation, indole-3-pyruvate decarboxylase, pyruvate decarboxylase, pyruvate decarboxylase (acetoin-forming)	Riboflavin metabolism, Biosynthesis of secondary metabolites
YDL080C	THI3	3-methyl-2-oxopentanoate decarboxylase, 4-methyl-2-oxopentanoate decarboxylase	Gluconeogenesis, Glycolysis, Biosynthesis of secondary metabolites, Biosynthesis of antibiotics
YER183C	FAU1	5-formyltetrahydrofolate cyclo-ligase, 5-formyltetrahydrofolate:10-formyltetrahydrofolate isomerase	One carbon pool by folate
YER037W YGR061C YIL107C YCR010C YIL160C	PHM8 ADE6 PFK26 ADY2 POT1	5'-nucleotidase (CMP), (UMP), (GMP), lysoPA phosphatase (16:0) (several lengths) 5'-phosphoribosylformyl glycaminidine synthetase 6-phosphofructo-2-kinase acetate transport, L-carnitine transport acetyl-CoA C-acyltransferase (palmitoyl-CoA and other Co-As)	Nicotinate and nicotinamide metabolism Purine metabolism, Biosynthesis of secondary metabolites, Biosynthesis of antibiotics Fructose and mannose metabolism Biosynthesis of amino acids Fatty acid degradation, Valine, leucine and isoleucine degradation, Biosynthesis of unsaturated fatty acids, Biosynthesis of secondary metabolites, Biosynthesis of antibiotics, Fatty acid metabolism, Peroxisome
YAL054C	ACS1	acetyl-CoA synthetase	Gluconeogenesis, Glycolysis, Pyruvate metabolism, Propionate metabolism, Biosynthesis of secondary metabolites, Biosynthesis of antibiotics, Carbon metabolism
YAR071W YER060W YER043C YDR226W	PHO11 FCY21 SAH1 ADK1	acid phosphatase (secreted) adenine transport, guanine transport, cytosine transport adenosylhomocysteinase adenylate kinase	Thiamine metabolism, Riboflavin metabolism, Cell cycle - yeast
YER170W	ADK2	adenylate kinase, adenylate kinase (GTP)	Cysteine and methionine metabolism Purine metabolism, Thiamine metabolism, Biosynthesis of secondary metabolites, Biosynthesis of antibiotics
YBL030C	PET9	ADP/ATP transporter	Purine metabolism, Thiamine metabolism, Biosynthesis of secondary metabolites, Biosynthesis of antibiotics

YMR303C	ADH2	alcohol dehydrogenase (ethanol to acetaldehyde)	Gluconeogenesis, Glycolysis, Fatty acid degradation, Tyrosine metabolism, Biosynthesis of secondary metabolites, Biosynthesis of antibiotics
YDL168W	SFA1	aldehyde dehydrogenase (2-methylbutanol, NAD) (2-phenylethanol, NAD) (isoamyl alcohol, NAD) (isobutyl alcohol, NAD) (tryptophol, NAD), formaldehyde dehydrogenase	Gluconeogenesis, Glycolysis, Fatty acid degradation, Tyrosine metabolism, Biosynthesis of secondary metabolites, Biosynthesis of antibiotics, Carbon metabolism
YDR481C	PHO8	alkaline phosphatase (dihydronoopterin)	Thiamine metabolism, Folate biosynthesis
YJR152W	DAL5	allantoate uniport, aminoacid transport via proton symport	Various types of N-glycan biosynthesis, Other types of O-glycan biosynthesis
YBR205W	KTR3	alpha 1,2-mannosyltransferase	Starch and sucrose metabolism
YDR074W	TPS2	alpha,alpha-trehalose-phosphate synthase (UDP-forming), trehalose-phosphatase	Galactose metabolism, Starch and sucrose metabolism
YIL172C	IMA3	alpha-glucosidase	Phenylalanine, tyrosine and tryptophan biosynthesis, Biosynthesis of secondary metabolites, Biosynthesis of antibiotics, Biosynthesis of amino acids
YDR384C	ATO3	ammonia transport	Phenylalanine, tyrosine and tryptophan biosynthesis, Biosynthesis of secondary metabolites, Biosynthesis of antibiotics, Biosynthesis of amino acids
YDR354W	TRP4	anthranilate phosphoribosyltransferase	Purine metabolism
YKL211C	TRP3	anthranilate synthase, indole-3-glycerol-phosphate synthase	Aminoacyl-tRNA biosynthesis
YDR305C	HNT2	Ap4A hydrolase	Glycine, serine and threonine metabolism, Monobactam biosynthesis, Cysteine and methionine metabolism, Lysine biosynthesis, Biosynthesis of secondary metabolites, Biosynthesis of antibiotics, 2-Oxocarboxylic acid metabolism, Biosynthesis of amino acids
YDR341C	YDR341C	arginyl-tRNA synthetase	Arginine biosynthesis, Alanine, aspartate and glutamate metabolism, Cysteine and methionine metabolism, Arginine and proline metabolism, Tyrosine metabolism, Phenylalanine metabolism, Phenylalanine, tyrosine and tryptophan biosynthesis, Biosynthesis of secondary metabolites, Biosynthesis of antibiotics, Carbon metabolism, 2-Oxocarboxylic acid metabolism, Biosynthesis of amino acids
YER052C	HOM3	aspartate kinase	Oxidative phosphorylation
YLR027C	AAT2	aspartate transaminase, tyrosine transaminase, L-erythro-4-hydroxyglutamate:2-oxoglutarate aminotransferase	Oxidative phosphorylation
YLL018C	DPS1	Aspartyl-tRNA synthetase	Oxidative phosphorylation
YJR121W	ATP2	ATP synthase	Oxidative phosphorylation
YBR039W	ATP3	ATP synthase	Oxidative phosphorylation
Q0085	ATP6	ATP synthase	Oxidative phosphorylation
Q0080	ATP8	ATP synthase	Oxidative phosphorylation
Q0130	OLI1	ATP synthase	Oxidative phosphorylation
YEL017C-A	PMP2	ATPase, cytosolic	Oxidative phosphorylation, Purine metabolism, Pyrimidine metabolism
YBR110W	ALG1	beta-1,4 mannosyltransferase	N-Glycan biosynthesis, Various types of N-glycan biosynthesis
YGL001C	ERG26	C-3 sterol dehydrogenase, C-3 sterol dehydrogenase (4-methylzymosterol)	Steroid biosynthesis, Biosynthesis of antibiotics
YLR056W	ERG3	C-5 sterol desaturase	Steroid biosynthesis, Biosynthesis of secondary metabolites, Biosynthesis of antibiotics
YJR109C	CPA2	carbamoyl-phosphate synthase (glutamine-hydrolysing)	Pyrimidine metabolism, Alanine, aspartate and glutamate metabolism
YER024W	YAT2	carnitine O-acetyltransferase	Peroxisome
YBR029C	CDS1	CDP-diacylglycerol synthase (1-16:0, 2-16:1), ER membrane	Glycerophospholipid metabolism, Biosynthesis of secondary metabolites, Phosphatidylinositol signaling system

YDR297W	SUR2	ceramide-1 hydroxylase (24C), ceramide-1 hydroxylase (26C), phytosphingosine synthesis	Sphingolipid metabolism
YKL008C	LAC1	ceramide-1 synthase (24C) (several lengths)	Sphingolipid metabolism
YLR133W	CKI1	choline kinase, ethanolamine kinase	Glycerophospholipid metabolism
YBR002C	RER2	cis-prenyltransferase step 01 to step 19	Terpenoid backbone biosynthesis, Biosynthesis of secondary metabolites
YCR005C	CIT2	citrate synthase, peroxisomal	Citrate cycle (TCA cycle), Glyoxylate and dicarboxylate metabolism, Biosynthesis of secondary metabolites, Biosynthesis of antibiotics, Carbon metabolism, 2-Oxocarboxylic acid metabolism, Biosynthesis of amino acids
YGR110W	CLD1	CL (1-16:0, 2-16:1, 3-16:0, 4-16:1) phospholipase (1-position), mitochondrial membrane (Multiple reactions with varying lengths)	Glycerophospholipid metabolism
YHR002W	LEU5	coenzyme A transport, mitochondrial membrane-cytoplasm	Cysteine and methionine metabolism, Selenocompound metabolism, Biosynthesis of secondary metabolites, Biosynthesis of amino acids
YGL184C	STR3	cystathionine b-lyase	Pantothenate and CoA biosynthesis
YDR196C	CAB5	dephospho-CoA kinase	Gluconeogenesis, Glycolysis, Pentose phosphate pathway, Fructose and mannose metabolism, Biosynthesis of secondary metabolites, Biosynthesis of antibiotics, Carbon metabolism, Biosynthesis of amino acids
YKL060C	FBA1	D-fructose 1-phosphate D-glyceraldehyde-3-phosphate-lyase, fructose-bisphosphate aldolase, sedoheptulose 1,7-bisphosphate D-glyceraldehyde-3-phosphate-lyase	Meiosis - yeast
YDL245C	HXT15	D-fructose transport D-mannose transport glucose transport D-sorbitol transport L-sorbitol transport L-sorbose transport xylitol transport D-tagatose uptake via diffusion	Meiosis - yeast
YDR345C, YHR096C, YDR343C, YDR342C	HXT3, HXT5, HXT6, HXT7	D-fructose transport D-mannose transport glucose transport D-sorbitol transport L-sorbitol transport L-sorbose transport xylitol transport D-tagatose uptake via diffusion	Meiosis - yeast
YEL069C	HXT13	D-fructose transport, D-mannose transport, glucose transport, D-sorbitol transport, L-sorbitol transport, L-sorbose transport, D-tagatose uptake via diffusion	Meiosis - yeast
YDL100C	GET3	dihydronicopterin monophosphate dephosphorylase	Valine, leucine and isoleucine biosynthesis, Pantothenate and CoA biosynthesis, Biosynthesis of secondary metabolites, Biosynthesis of antibiotics, 2-Oxocarboxylic acid metabolism, Biosynthesis of amino acids
YJR016C	ILV3	dihydroxy-acid dehydratase (2,3-dihydroxy-3-methylpentanoate)	Fatty acid elongation, Biosynthesis of unsaturated fatty acids, Biosynthesis of secondary metabolites, Fatty acid metabolism
YJL196C	ELO1	elongase I (3-oxotetradecanoyl-CoA), elongase I (3-oxopalmitoyl-CoA)	Pentose and glucuronate interconversions
YJR153W	PGU1	endopolygalacturonase	Sulfur metabolism
YKR066C	CCP1	ferrocytochrome-c:hydrogen-peroxide oxidoreductase	Oxidative phosphorylation, Sulfur metabolism
Q0045	COX1	ferrocytochrome-c:oxygen oxidoreductase	Oxidative phosphorylation, Sulfur metabolism
Q0250	COX2	ferrocytochrome-c:oxygen oxidoreductase	Oxidative phosphorylation, Sulfur metabolism
Q0275	COX3	ferrocytochrome-c:oxygen oxidoreductase	Oxidative phosphorylation, Sulfur metabolism
YIL111W	COX5B	ferrocytochrome-c:oxygen oxidoreductase	Oxidative phosphorylation, Sulfur metabolism
YLR011W	LOT6	FMN reductase	Fructose and mannose metabolism
YJL155C	FBP26	fructose-2,6-bisphosphate 2-phosphatase	

YDR300C	PRO1	glutamate 5-kinase	Arginine and proline metabolism, Carbapenem biosynthesis, Biosynthesis of antibiotics, Biosynthesis of amino acids
YDL215C	GDH2	glutamate dehydrogenase (NAD)	Arginine biosynthesis, Alanine, aspartate and glutamate metabolism, Taurine and hypotaurine metabolism, Nitrogen metabolism
YKL104C	GFA1	glutamine-fructose-6-phosphate transaminase	Alanine, aspartate and glutamate metabolism, Amino sugar and nucleotide sugar metabolism, Biosynthesis of antibiotics
YGL245W	GUS1	glutamyl-tRNA synthetase	Porphyrin and chlorophyll metabolism, Aminoacyl-tRNA biosynthesis, Biosynthesis of secondary metabolites
YIR037W	HYR1	glutathione peroxidase	Glutathione metabolism
YJL212C	OPT1	glutathione transport, oxidized glutathione uniport	Gluconeogenesis, Glycolysis, Biosynthesis of secondary metabolites, Biosynthesis of antibiotics, Carbon metabolism, Biosynthesis of amino acids
YJR009C	TDH2	glyceraldehyde-3-phosphate dehydrogenase	Glycerolipid metabolism
YHL032C	GUT1	glycerol kinase	Glycerolipid metabolism
YGL084C	GUP1	glycerol transport	Glycerolipid metabolism, Glycerophospholipid metabolism, Biosynthesis of secondary metabolites
YKR067W	GPT2	glycerol-3-phosphate acyltransferase (16:0), ER membrane, dihydroxyacetone phosphate acyltransferase (16:0), ER membrane, glycerol-3-phosphate acyltransferase (16:0), lipid particle, dihydroxyacetone phosphate acyltransferase (16:0), lipid particle (all in several lengths)	Glycerophospholipid metabolism, Biosynthesis of secondary metabolites, MAPK signaling pathway - yeast
YDL022W	GPD1	glycerol-3-phosphate dehydrogenase (NAD)	Glycine-cleavage complex (lipoamide), oxoglutarate dehydrogenase (dihydrolipoamide S-succinyltransferase), oxoglutarate dehydrogenase (lipoamide)
YDR148C	KGD2	glycine-cleavage complex (lipoamide), oxoglutarate dehydrogenase (dihydrolipoamide S-succinyltransferase), oxoglutarate dehydrogenase (lipoamide)	Purine metabolism, Biosynthesis of secondary metabolites, Carbon metabolism
YJR013W	GPI14	GPI-anchor assembly, step 5	Glycosylinositol-phosphate (GPI)-anchor biosynthesis
YCL052C	PBN1	GPI-anchor assembly, step 5	Glycosylinositol-phosphate (GPI)-anchor biosynthesis
YDL198C	GGC1	GTP/GDP translocase	Histidine metabolism, Biosynthesis of secondary metabolites, Biosynthesis of amino acids
YDR399W	HPT1	guanine phosphoribosyltransferase, hypoxanthine phosphoribosyltransferase (Hypoxanthine)	Histidine metabolism, Tyrosine metabolism, Phenylalanine metabolism, Phenylalanine, tyrosine and tryptophan biosynthesis, Biosynthesis of secondary metabolites, Biosynthesis of antibiotics, Biosynthesis of amino acids
YCL030C	HIS4	histidinol dehydrogenase, phosphoribosyl-AMP cyclohydrolase, phosphoribosyl-ATP pyrophosphatase	Lysine biosynthesis, Pyruvate metabolism, Biosynthesis of antibiotics, 2-Oxocarboxylic acid metabolism, Biosynthesis of amino acids
YIL116W	HIS5	histidinol-phosphate transaminase	Histidine metabolism, Biosynthesis of secondary metabolites, Biosynthesis of amino acids
YBR034C	HMT1	hnRNP arginine N-methyltransferase	Histidine metabolism, Biosynthesis of secondary metabolites, Biosynthesis of amino acids
YDL131W	LYS21	homocitrate synthase	Histidine metabolism, Biosynthesis of secondary metabolites, Biosynthesis of amino acids
YDR453C	TSA2	hydrogen peroxide reductase (thioredoxin)	Histidine metabolism, Biosynthesis of secondary metabolites, Biosynthesis of amino acids
YBR248C	HIS7	Imidazole-glycerol-3-phosphate synthase	

YBR011C	IPPI	inorganic diphosphatase	Oxidative phosphorylation
YDR497C	ITR1	inositol transport	Inositol phosphate metabolism, Phosphatidylinositol signaling system
YDR315C	IPK1	inositol-1,3,4,5,6-pentakisphosphate 2-kinase	
YKL004W	AUR1	IPC synthase (PI (1-16:0, 2-16:1) ceramide-1 (C24)) (Multiple reactions with varying lengths)	
YER019W	ISC1	IPS phospholipase C	Sphingolipid metabolism
YER065C	ICL1	isocitrate lyase	Glyoxylate and dicarboxylate metabolism, Biosynthesis of secondary metabolites, Carbon metabolism
YBR115C	LYS2	L-amino adipate-semialdehyde dehydrogenase (NADPH)	Lysine biosynthesis, Pantothenate and CoA biosynthesis, Biosynthesis of secondary metabolites, Biosynthesis of antibiotics, Biosynthesis of amino acids
YHR072W	ERG7	lanosterol synthase	Steroid biosynthesis, Biosynthesis of secondary metabolites, Biosynthesis of antibiotics
YEL063C	CAN1	L-arginine transport, ornithine transport	
	ASP1	L-asparaginase	
YDR508C	GNP1	L-asparagine transport, L-cystine transport, L-glutamine transport, L-leucine transport, L-methionine transport, L-threonine transport, L-serine transport, asparagine transport, methionine transport, threonine transport, serine transport	Alanine, aspartate and glutamate metabolism, Biosynthesis of secondary metabolites
YCR075C	ERS1	L-cystine transport	
YGR055W	MUP1	L-methionine transport	
YJR078W	BNA2	L-tryptophan:oxygen 2,3-oxidoreductase (decyclizing)	Tryptophan metabolism
YBR042C	CST26	lysoPI acyltransferase (1-18:0, 2-16:1), ER membrane	
YKL085W	MDH1	malate dehydrogenase	Citrate cycle (TCA cycle), Cysteine and methionine metabolism, Pyruvate metabolism, Glyoxylate and dicarboxylate metabolism, Biosynthesis of secondary metabolites, Biosynthesis of antibiotics, Carbon metabolism
			One carbon pool by folate, Aminoacyl-tRNA biosynthesis
			Selenocompound metabolism, Aminoacyl-tRNA biosynthesis
YBL013W	FMT1	methionyl-tRNA formyltransferase	
YGR264C	MES1	methionyl-tRNA synthetase	
YBR180W	DTR1	N,N'-diformyltyrosine transport	
YFL017C	GNA1	N-acetylglucosamine-6-phosphate synthase	Amino sugar and nucleotide sugar metabolism
YGL067W	NPY1	NAD diphosphatase	Nicotinate and nicotinamide metabolism, Peroxisome
YDR191W	HST4	NAD nucleosidase nuclear	Nicotinate and nicotinamide metabolism, Longevity regulating pathway - multiple species
YEL006W	YEA6	NAD transport	
YIL006W	YIA6	NAD transport	
YDR428C	BNA7	N-formyl-L-kynurenine amidohydrolase	Tryptophan metabolism, Glyoxylate and dicarboxylate metabolism
YGR010W	NMA2	nicotinamide-nucleotide adenylyltransferase, nicotinate-nucleotide adenylyltransferase	Nicotinate and nicotinamide metabolism
YEL021W	URA3	orotidine-5'-phosphate decarboxylase	Pyrimidine metabolism
YIL145C	PAN6	pantothenate synthase	beta-Alanine metabolism, Pantothenate and CoA biosynthesis, Biosynthesis of secondary metabolites
YLR060W	FRS1	phenylalanyl-tRNA synthetase	Aminoacyl-tRNA biosynthesis
YFL022C	FRS2	phenylalanyl-tRNA synthetase	Aminoacyl-tRNA biosynthesis
YGL234W	ADE_7	phosphoribosylaminoimidazole synthetase, phosphoribosylglycinamide synthetase	Purine metabolism, Biosynthesis of secondary metabolites, Biosynthesis of antibiotics
YCR037C	PHO87	phosphate transport	

YJL198W	PHO90	phosphate transport	
YGR007W	ECT1	phosphoethanolamine cytidyltransferase	Phosphonate and phosphinate metabolism, Glycerophospholipid metabolism
YKL127W	PGM1	phosphoglucomutase, phosphopentomutase	Gluconeogenesis, Glycolysis, Pentose phosphate pathway, Galactose metabolism, Purine metabolism, Starch and sucrose metabolism, Amino sugar and nucleotide sugar metabolism, Biosynthesis of secondary metabolites, Biosynthesis of antibiotics
YCR012W	PGK1	phosphoglycerate kinase	Gluconeogenesis, Glycolysis, Biosynthesis of secondary metabolites, Biosynthesis of antibiotics, Carbon metabolism, Biosynthesis of amino acids
YKR072C	SIS2	phosphopantethenoylcysteine decarboxylase	Pantothenate and CoA biosynthesis
YAR015W	ADE1	phosphoribosyl amino imidazolesuccinocarbozamide synthetase	Purine metabolism, Biosynthesis of secondary metabolites, Biosynthesis of antibiotics
YDR007W	TRP1	phosphoribosylanthranilate isomerase	Phenylalanine, tyrosine and tryptophan biosynthesis, Biosynthesis of secondary metabolites, Biosynthesis of antibiotics, Biosynthesis of amino acids
YER026C	CHO1	PS synthase (1-16:0, 2-16:1), ER membrane (several lengths)	Glycine, serine and threonine metabolism, Glycerophospholipid metabolism, Biosynthesis of secondary metabolites
YBR221C	PDB1	pyruvate dehydrogenase	Gluconeogenesis, Glycolysis, Citrate cycle (TCA cycle), Glycine, serine and threonine metabolism, Valine, leucine and isoleucine degradation, Pyruvate metabolism, Glyoxylate and dicarboxylate metabolism, Propanoate metabolism, Biosynthesis of secondary metabolites, Biosynthesis of antibiotics, Carbon metabolism
YGR193C	PDX1	pyruvate dehydrogenase	Gluconeogenesis, Glycolysis, Citrate cycle (TCA cycle), Glycine, serine and threonine metabolism, Valine, leucine and isoleucine degradation, Pyruvate metabolism, Glyoxylate and dicarboxylate metabolism, Propanoate metabolism, Biosynthesis of secondary metabolites, Biosynthesis of antibiotics, Carbon metabolism
YGL080W	MPC1	pyruvate transport	
YIL020C	HIS6	raffinose invertase, 1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)methylideneamino)4-carboxamide isomerase	Raffinose metabolism, Biosynthesis of secondary metabolites, Biosynthesis of amino acids
YBR256C	RIB5	riboflavin synthase	Riboflavin metabolism, Biosynthesis of secondary metabolites
YBR132C	AGP2	spermidine transport	
YKR053C	YSR3	sphingoid base-phosphate phosphatase (phytosphingosine 1-phosphate), sphingoid base-phosphate phosphatase (sphinganine 1-phosphatase)	Sphingolipid metabolism
YJL045W	YJL045W	succinate dehydrogenase (ubiquinone-6)	Citrate cycle (TCA cycle), Oxidative phosphorylation, Biosynthesis of secondary metabolites, Biosynthesis of antibiotics, Carbon metabolism
YIL162W	SUC2	sucrose hydrolyzing enzyme	Galactose metabolism, Starch and sucrose metabolism
YJR137C	MET5	sulfite reductase (NADPH2)	Sulfur metabolism
YMR313C	TGL3	TAG lipase (1-16:0, 2-16:1, 3-16:0), lipid particle, DAG lipase (1-16:0, 2-16:1), lipid particle, lysoPE (1-16:0) oleoyl-CoA acyltransferase, lipid particle (all in several lengths)	Steroid biosynthesis, Glycerolipid metabolism, Glycerophospholipid metabolism, Biosynthesis of secondary metabolites
YKL132C	RMA1	tetrahydrofolate:L-glutamate gamma-ligase (ADP-forming)	Folate biosynthesis
YGR096W	TPC1	thiamine diphosphate transport	
YDL015C	TSC13	trans-2-enoyl-CoA reductase (n-C14:0CoA) (several lengths)	Fatty acid elongation, Biosynthesis of unsaturated fatty acids, Biosynthesis of secondary metabolites, Fatty acid metabolism

YGL026C	TRP5	tryptophan synthase (indoleglycerol phosphate)	Glycine, serine and threonine metabolism, Phenylalanine, tyrosine and tryptophan biosynthesis, Biosynthesis of secondary metabolites, Biosynthesis of antibiotics, Biosynthesis of amino acids
Q0105	COB	ubiquinol:ferricytochrome c reductase	Oxidative phosphorylation, Sulfur metabolism
YJL166W	QCR8	ubiquinol:ferricytochrome c reductase	Oxidative phosphorylation, Sulfur metabolism
YEL024W	RIP1	ubiquinol:ferricytochrome c reductase	Oxidative phosphorylation, Sulfur metabolism
YDL103C	QRI1	UDP-N-acetylglucosamine diphosphorylase	Amino sugar and nucleotide sugar metabolism, Biosynthesis of antibiotics
YBL042C	FUI1	uridine transport, deoxyuridine transport	
YKL035W	UGP1	UTP-glucose-1-phosphate uridylyltransferase	Pentose and glucuronate interconversions, Galactose metabolism, Starch and sucrose metabolism, Amino sugar and nucleotide sugar metabolism, Biosynthesis of antibiotics
YJR148W	BAT2	valine transaminase, leucine transaminase, isoleucine transaminase, 2-oxo-4-methyl-3-carboxypentanoate decarboxylation, 2-keto-4-methylthiobutyrate transamination	Cysteine and methionine metabolism, Valine, leucine and isoleucine degradation, Valine, leucine and isoleucine biosynthesis, Pantothenate and CoA biosynthesis, Biosynthesis of secondary metabolites, Biosynthesis of antibiotics, 2-Oxocarboxylic acid metabolism, Biosynthesis of amino acids
YLL052C	AQY2	water diffusion, H <sub>2</sub> O transport, 3-dehydroquinate dehydratase, 3-dehydroquinate synthase, 3-phosphoshikimate 1-carboxyvinyltransferase, shikimate dehydrogenase, shikimate kinase	
YJR133W	XPT1	xanthine phosphoribosyltransferase	
YFL001W	DEG1	yUMP synthetase	
YKL152C	GPM1		Gluconeogenesis, Glycolysis, Glycine, serine and threonine metabolism, Biosynthesis of secondary metabolites, Biosynthesis of antibiotics, Carbon metabolism, Biosynthesis of amino acids
YCR036W	RBK1		Pentose phosphate pathway

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## APPENDIX A: APPLICATION

The appendices start here. After references section.