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Model-driven design of a *Saccharomyces cerevisiae* platform strain with improved tyrosine production capabilities

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Abstract: *Saccharomyces cerevisiae*, a eukaryotic model organism, is considered the ideal host for microbial production of plant secondary metabolites such as polyketides and alkaloids. However, industrial scale production of these valuable products using *S. cerevisiae* is limited by the availability of their precursor, aromatic amino acid tyrosine. Here, we describe a framework which uses a combination of computational modeling techniques to design an *in silico* metabolic engineering strategy that improves the flux through the aromatic amino acid pathway (shikimate pathway) in *S. cerevisiae*. The predicted yeast strain can be used as a platform strain for production of any heterologous products which require tyrosine, or any other aromatic amino acid pathway metabolites as precursors. The initial genome-scale strain design was performed using steady-state constraint-based modeling methods, Optknock and GDLS. The resulting design required deletion of multiple genes and was difficult to validate experimentally. In order to obtain an experimentally feasible design, a small-scale kinetic model was developed using Ensemble Modeling, and was used to prioritize the knockouts predicted by steady-state models for experimental validation.

Keywords: Saccharomyces cerevisiae, metabolic engineering, computational models, polyketides, aromatic amino acids

1. INTRODUCTION

Plant secondary metabolites such as polyketides and alkaloids are a diverse and important class of naturally-derived compounds with applications in pharmaceuticals, nutrition, and flavouring. Large-scale production of these valuable plant natural products is limited by the slow growth rate of some of the producing plants and typically low production efficiencies. Chemical synthesis of these compounds is challenging because of their complex structures. Therefore, there is a great incentive for producing these valuable compounds using microbial routes, which can facilitate industrial-scale production. Recent advances in recombinant DNA technology and synthetic biology have allowed for heterologous production of important plant secondary metabolites in genetically modified micro organisms (Maury et al., 2005, Hawkins et al 2008; Yan et al., 2007; Facchini et al., 2012).

The yeast, *S. cerevisiae* is considered the ideal choice for heterologous production of polyketides and alkaloids primarily because its eukaryotic nature facilitates functional expression of plant derived enzymes of the alkaloid and polyketide pathways (Primrose, 1986; Zabriskie et al., 1986). In addition, the experimental techniques required to effectively express non-native plant genes are widely available for *S. cerevisiae*. Finally, the GRAS (generally regarded as safe) status of *S. cerevisiae* is attractive considering the nutrition and pharmaceutical applications of polyketides and alkaloids.

Although several studies (Porro et al., 2005; Dejong et al., 2006; Ro et al., 2006) have reported that production of plant secondary metabolites in *S. cerevisiae* is feasible, their yields are limited by insufficient supply of precursor metabolites (Jiang et al., 2005). As a consequence, the current microbial production processes of important plant products require addition of expensive precursor metabolites as media supplements, which can prove to be a major hurdle for economic feasibility. Therefore, for economically feasible industrial-scale production of these bioproducts, it is necessary to increase the flux towards microbial precursors.

In this study, the objective is to obtain a *S. cerevisiae* "platform strain" with improved tyrosine pools which can be used as a microbial host for production of important compounds such as vinblastine, strictosidine, morphine, codeine etc. In yeast, like other organisms, tyrosine is produced using shikimate pathway which starts from the central carbon metabolites phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E4P). There is limited work done previously on improving the aromatic amino acid production in *S. cerevisiae* as *E. coli* is the preferred microbe for industrial-scale production of aromatic amino acids. To our knowledge, Luttik et al.,(2008) and more recently Koopman et al., (2012) are the only reported works that focused on engineering *S. cerevisiae* for over-production of aromatic amino acids. Their works described the importance of removing the feedback inhibition in the shikimate pathway and disrupting the aromatic amino acid degradation pathways on improving the intracellular aromatic amino acid pools.

While the above mentioned efforts did result in improvement of the aromatic amino acid pools, their engineering strategies were limited only to the shikimate pathway. However, to maximize the production of tyrosine, a holistic design that would account for the entire genome of the yeast is required. The process of performing a genome-scale design is not trivial and cannot be performed by observation because of the complexity of metabolic networks. This complexity of metabolic networks acts as a motivation for using computational modeling techniques for designing metabolic engineering strategies. In this work, we describe a methodology which uses a combination of steady-state and dynamic modeling techniques to design an effective engineering strategy for improving tyrosine production in *S. cerevisiae*.

2. METHODS

2.1 Steady-state modeling

In this study, initial strain design was performed using steady-state strain design algorithms over the *S. cerevisiae* genome-scale model iMM904 (Mo et al., 2009). Bilevel optimization methods, Optknock (Burgard et al., 2003) and GDLS (Genetic Design by Local Search) (Lun et al., 2009), which are based on the fundamental steady-state modeling methodology called Flux Balance Analysis (FBA) (Orth et al., 2010) were used for steady-state strain design. The OptKnock strain design algorithm was performed using an in-house implementation, searching up to four simultaneous reaction deletions with the bioengineering objective of maximizing an artificial cytosolic L-tyrosine exchange flux and the cellular objective of growth rate maximization. The simulation was done using glucose as the limiting substrate under aerobic minimal media conditions. Genetic Design by Local Search (GDLS) (Lun DS, 2009) was performed using an in-house implementation with the same boundary and media conditions and either cytosolic L-tyrosine or cytosolic chorismate exchange fluxes as the bioengineering objective. GDLS was run with a neighbourhood size of 2 and a maximum of 10 knockouts. Steady-state simulations were done in Matlab using CPLEX ILOG for optimization.

2.2 Dynamic modeling

In this work, dynamic modeling approach was used to prioritize the knockout predictions made by steady-state modeling for experimental validation. The kinetic models of *S. cerevisiae* reported in literature are currently restricted to the central carbon metabolism (Rizzi et al., 1997; Teusink et al., 2000). These models could not be used for our purpose because their reaction networks did not include the target reactions suggested by steady-state models. Therefore, a small central model which could estimate the dynamic behaviour of *S. cerevisiae* was developed using Ensemble modeling (EM) procedure reported by Tran et al., (2008). Ensemble modeling has been used previously to improve the yields of lysine in *Escherichia coli* (Contador et al., 2009).

2.2.1 Development of ensemble models

The framework for ensemble modeling requires the reaction network, reference steady-state flux data, reference steady-state concentration data and the Gibbs free energy (ΔGs) values for reactions as the input to generate the models. A network with sixty three reactions that includes the reactions from central carbon metabolism of *S. cerevisiae* and also the reactions suggested by steady-state modeling (Fig. 1) was designed. Compartmentalisation in *S. cerevisiae* was accounted by separating the metabolites in cytoplasm and mitochondria. Metabolites that are present in both the compartments were connected through exchange reactions. However, cofactors like ATP, NADPH and NADH were assumed to freely transport across the two compartments. The ΔGs for the reactions in the network were obtained from Jankowski et al. (2008).

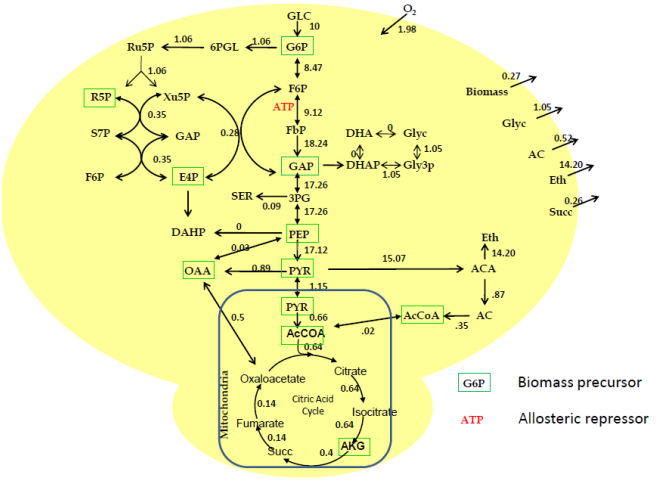

Fig. 1: Schematic of *S. cerevisiae* central model used in this work along with calculated steady-state flux data. All reported fluxes are in (mmol/gDwhr).

EM framework uses a reference steady-state flux data to anchor the models. The reference steady-state flux data for the network was estimated from the experimental C13 flux data (Blank et al., 2005) using the following optimization formulation

$$\begin{aligned} &\textit{Minimize} && (V_{cal} - V_m)^2 \\ &\textit{Subject to} && S * V_{cal} = 0 \\ & && 0 < V_{cal,irrev} < 1000 \\ & && -1000 < V_{cal,\,rev} < 1000 \end{aligned}$$

In the above formulation, V_{cal} is the calculated steady-state flux data for all the reactions in the network and V_m is the corresponding reported flux value in Blank et al (2005). All flux values in the formulation have the units of (mmol/gDwhr). S is the stoichiometric matrix. The reaction bounds are chosen as 0 and 1000 for irreversible ($V_{cal,irrev}$), and -1000 and 1000 for reversible reactions ($V_{cal,rev}$). The biomass equation for the reconstructed model was obtained from Heer et al. (2009), and was assumed to be composed of:

1.911 G6P[c] + 0.351 R5P[c] + 0.363 GAP[c] + 1.332 OAA[c] + 0.584 SER[c] + 1.397 ACCOA[c] + 0.155 ACCOA[m] + 0.997 OXOGLUTARATE[m] + 2.147 PYR[m] + 0.239 PYR[c] + 0.579 PEP[c] + 0.289 E4P[c] + 11.352 ATP + 11.249 NADPH + 0.118 NAD ---> Biomass + 11.249 NADP + 0.118 NADH + 11.352 ADP

[c] = cytoplasmic and [m] = mitochondrial

As the information on reference steady-state intracellular metabolite concentrations was not available, we assumed all internal metabolites were at a concentration of one mmol at steady-state. Under this assumption, the concentrations of metabolites in each model were scaled by the corresponding concentration at the reference steady-state. EM can also account for allosteric regulation. In our network, the reported allosteric repression of Fructose-6-Phosphate (F6P) to Fructose-1,6-bis-Phosphate (FbP) by ATP (Simonis et al., 2004) was considered. Using the above inputs a set of 2500 models were generated using the Matlab module previously developed (http://www.seas.ucla.edu/~liaoj). Each of these 2500 models had a different set of kinetic parameter values, but all of them were anchored to the same steady-state flux data.

2.2.2 Screening the ensemble using data from literature

The 2500 generated models were then screened using the data from glycerol (Nevoigt et al., 1996) and succinic acid (Raab et al., 2010) over-producing strains reported in the literature. These data sets were chosen because the modifications done in these studies were performed on reactions that were included in our central model. The EM framework applied in this work is qualitative, and cannot be expected to give an accurate prediction of the observed experimental flux data. Therefore, while screening the models, a 25% error range was allowed on the predicted flux values.

In the glycerol over-producing strain, when pyruvate decarboxylase (PDC--) gene was repressed to approximately 20% activity, the yield of glycerol increased by 4.5-folds. When glycerol-3-phosphate dehydrogenase (GPD++) was over-expressed by increasing its activity 20-fold, six fold increase in the yield of glycerol was observed, and when these two manipulations(PDC-- and GPD++) were performed simultaneously, the glycerol yield increased by approximately 8-fold. In each of the above mentioned glycerol over-production cases, ethanol production rate decreased. In the succinate over-producing strain, when succinate dehydrogenase (ΔSDH) and isocitrate dehydrogenase (ΔIDH) genes were deleted an increase in succinate production, along with 25% reduction in the growth rate of the strain, was observed.

For model screening, each of the 2500 models was pertured computationally by modifying the *in silico* enzyme concentration to reflect the genetic manipulation performed experimentally, and the models that predicted the experimental observation within the allowed error range were selected (Fig. 2). Out of the 2500 models 146 and 317 models were able to qualitatively depict the experimental observations in the glycerol and succinate strains respectively and 42 models were able to depict both the strains. This set of 42 models, assumed to better represent the dynamic behaviour of *S. cerevisiae*, were used to predict how the flux distribution changes with deletion of reactions suggested from steady-state modeling.

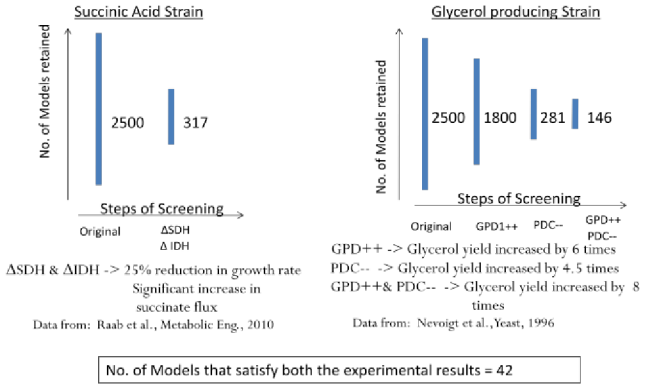

Fig. 2: Screening of the 2500 models using data from succinic acid and glycerol over-producing strains.

2.3 Experimental procedure for ARO10 deletion studies

In this study, four strains of *S. cerevisiae*: wild-type (WT), wild-type with ARO4 feedback insensitive gene ($ARO4^{FBR}$), ARO10 deletion mutant (ΔARO10), and the ARO10 deletion mutant with ARO4 feedback insensitive gene (ΔARO10+$ARO4^{FBR}$) were compared for their ability to accumulate coumarate, the sink for tyrosine, during fermentation. Accumulation of tyrosine cannot be directly monitored because it is not exported out in *S. cerevisiae*. ARO10 deletion was implemented in the triple auxotroph wild-type strain, CEN.PK111-61A (ura^-, leu^-,his^-) through homologous recombination, using hygromycin resistance marker-containing disruption cassette created by PCR, as described by Gueldener et al. (2002a, 2002b). Integration cassette contained two 40-nt regions of homology corresponding to the 5' and 3' ends of the target locus. The loxLE/RE-flanked hphNT1 cassette was amplified from the pZC3 vector (Carter and Delneri, 2010; Gueldener et al., 2002b).

Tyrosine ammonia lyase (TAL), the gene that converts tyrosine to coumarate, is not a native gene in *S. cerevisiae*. Therefore, to monitor coumarate levels, the TAL gene needs to be transformed into *S. cerevisiae*. TAL from *Rhodobacter sphaeroides* was synthesized and codon-optimized by DNA 2.0 (Menlo Park, CA, USA) and was assembled into a leucine selective, 2μ shuttle vector derived from pYES2 (Life Technologies, Carlsbad, CA, USA). The feedback inhibition resistant version of the 3-deoxy-d-arabinose-heptulosonate-7-phosphate (DAHP) synthase ARO4 was obtained by introducing the K229L mutation by PCR, using *S. cerevisiae* CEN.PK genomic DNA as template (Luttik et al., 2008). This feedback insensitive ARO4 gene was cloned into a uracil selective pGREG506 vector (Jansen et al., 2005). Plasmid with TAL was present in all four strains while the plasmid with ARO4 feedback insensitive gene was there in only $ARO4^{FBR}$ and ΔARO10+$ARO4^{FBR}$ strains. The plasmids

were transformed using Lithium Acetate transformation protocol (Gietz et al., 1995).

All strains were grown in triplicates at 30 °C and 150 rpm on defined SD medium (Bergman, 2001) supplemented with amino acids to complement specific auxotrophic requirements (Sherman, 2002) for five days. Samples were collected every twenty four hours and were analysed for coumarate on an Eclipse XDB-C18 column (4.6×150mm, 5µm, Agilent), using an Agilent 1200 HPLC system.

3. RESULTS AND DISCUSSION

3.1 Steady-state modeling results

Steady-state strain design algorithms are used to identify deletions in a metabolic network that could improve desired metabolite yield, which in our case is tyrosine. The targets for deletions are reactions, which when deleted will force the resulting mutant strain to produce the desired metabolite in order to reach its optimal growth rate, a scenario known as "growth-coupled." The advantage of this modeling mechanism is that it can be used for genome-scale design because of the minimal amount of biological knowledge and data it requires to predict strategies. Similar approach has been previously used to improve production of a variety of products in *S. cerevisiae* (Asadollahi et al., 2009; Bro et al., 2006; Brochado et al., 2010).

Our initial attempt at steady-state strain design was performed using Optknock over iMM904. Optknock uses a global search approach while designing metabolic engineering strategies. This global search approach is computationally intensive and is therefore limited to searching a relatively small number of simultaneous knockouts in genome-scale models. Optknock was not able to find a growth-coupled solution for tyrosine even after searching up to four knockouts, and did not converge when the maximum knockout limit was increased above four.

In order to expand the search for a higher number of knockouts, local search algorithm GDLS, which has similar formulation as Optknock, was used. When GDLS was applied with tyrosine production as the bioengineering objective, it did not yield any solution. As GDLS is a local search algorithm, the path followed by the algorithm while performing a search can be critical. To direct the algorithm path, a strain that maximizes for chorismate production was initially designed. Chorismate is a metabolite in shikimate pathway and a precursor to all the three aromatic amino acids. The chorismate strain, instead of wild-type iMM904, was used as the seed for tyrosine design using GDLS. This approach resulted in a successful strain design that required deletion of seven reactions: three reactions in glycolysis – gly1, gly2, gly3; two reactions in TCA cycle – tca1, tca2; a reaction in pentose phosphate pathway and aromatic amino acids degradation reaction.

FBA simulation of this strategy, performed using COBRA tool box (Becker et al., 2007) over iMM904 with glucose and oxygen uptake rates of 10(mmol/gDwhr) under minimal media conditions, showed excellent growth coupling (Fig. 3). From Fig. 3, it is clear that while there is no growth coupling of tyrosine production for wild type strain, the designed mutant strain is forced to produce tyrosine. Furthermore, according to our hypothesis, cells should grow at a state where growth rate is maximum because maximizing biomass was the cellular objective. At this state of maximum growth rate, which for wild-type is at A in Fig. 3, FBA predicts no production of tyrosine in wild-type as expected. However, in the case of the mutant, after adaptive evolution, the mutant is expected to grow at B in Fig. 3, where it is predicted to produce tyrosine at 60% of the theoretical yield.

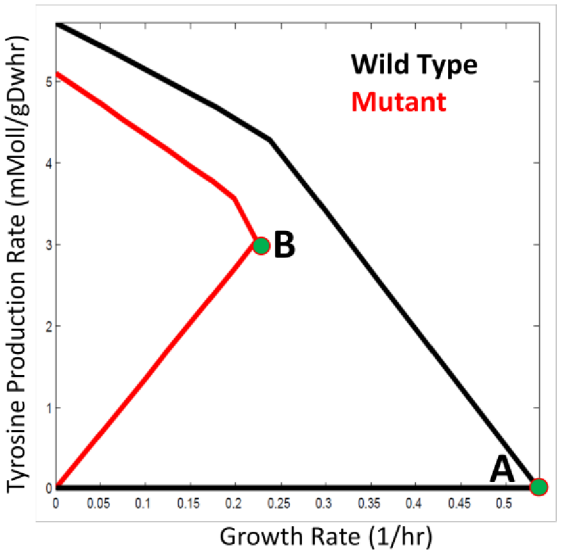

Fig. 3: Map of FBA predicted solution space for wild-type iMM904 and the mutant designed from GDLS. A and B represent the optimal growth points for wild-type and mutant.

3.2 Experimental validation for ARO10 deletion

ARO10 gene, which is responsible for the degradation of aromatic amino acids, was chosen as the first target for experimental validation. The result of ARO10 deletion mutant was compared with the previously reported work that focused on removal of feedback inhibition in the shikimate pathway of *S. cerevisiae* (Luttik et al., 2008). The accumulation of coumarate, the sink for tyrosine, was monitored for four strains: WT, ARO4^{FBR}, ΔARO10, ΔARO10+ARO4^{FBR} (Fig. 4).

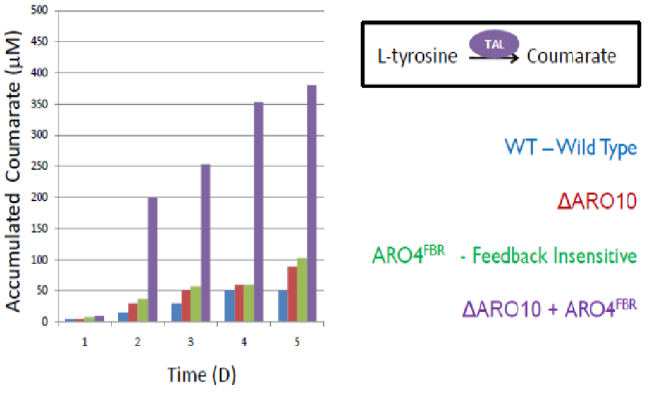

Fig. 4: Accumulation of coumarate, the sink for tyrosine, in the four strains that were compared.

From the above data it can be observed that, accumulation rates of coumarate in model suggested ΔARO10 mutant were comparable to that of $ARO4^{FBR}$ mutant reported earlier by Luttik et al. However, when removal of feedback inhibition was combined with ARO10 gene deletion, which is the $\Delta ARO10+ARO4^{FBR}$ mutant, the intracellular levels coumarate were much higher than in either of the above cases. Recently Koopman et al., (2012), working on production of naringenin in *S. cerevisiae,* also made similar observations.

While the ARO10 deletion study indicated the importance of using steady-state models for designing metabolic engineering strategies, there are many limitations to the practical implementation of strain designs obtained using these models. In particular, because regulatory and metabolite concentration information is not accounted in such models, many of the design suggestions are either unnecessary or biologically infeasible. For example, in this study the model predicts that seven deletions are required for completely growth-coupled tyrosine production. However, it is experimentally tedious, and possibly even lethal, to make a mutant with seven gene deletions. Therefore, it is important to determine which of the deletions suggested by the steady-state model are more effective in re-routing the carbon flux towards tyrosine, while not having an adverse effect on growth. In order to address this, we employed kinetic models of metabolism to verify the effects of mutations discovered by genome-scale strain design algorithms.

3. 3 Dynamic modeling results

In order to determine the most effective subset of reactions suggested by steady-state models, the 42 models screened from EM were used to make an *in silico* prediction of the metabolic flux distribution when each of the remaining six reactions other than ARO10 are deleted. A time period of 1000 seconds and a step size of 50 was used while simulating the deletions in 42 models. The effect of these deletions on biomass formation, accumulation of shikimate pathway precursors PEP, E4P and also on the accumulation of DAHP, the first metabolite of shikimate pathway was monitored (Fig. 5). In our dynamic model the entire aromatic amino acid pathway was not included to reduce the size of the network and considered DAHP as a proxy metabolite for the entire pathway (Fig. 1).

The plots below (Fig. 5) show average rate of accumulation of PEP, E4P, DAHP and biomass formation predicted for the 42 models. Based on the above data two deletions were selected, one in glycolysis – gly1 and one in pentose phosphate pathway – ppp, as the targets for subsequent experimental validation. The selections were based on the ability to increase the shikimate pathway precursor pools, PEP and E4P and that of DAHP, while not being lethal to cell growth. The actual reaction names were kept confidential in this paper because the experimental implementation of these deletions is currently in progress.

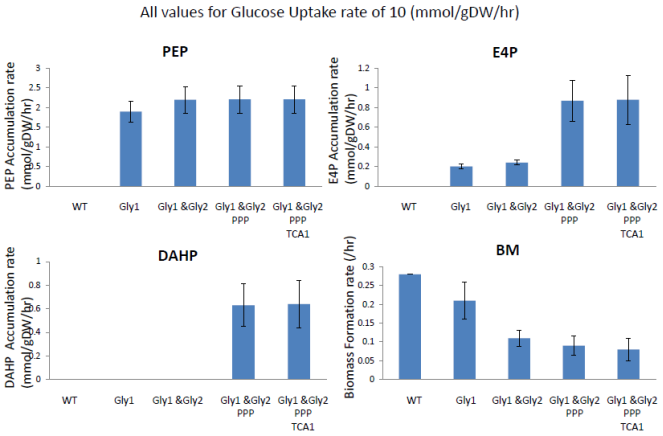

Fig. 5: Estimation of average PEP, E4P, DAHP accumulation and biomass formation rates when deletions suggested by GDLS are implemented using the forty two dynamic models obtained from Ensemble modeling.

4. CONCLUSION

This work is the first reported study that investigated the genome-wide engineering of *S. cerevisiae* for improved tyrosine production. Although steady-state strain design algorithms are effective in predicting genetic engineering strategies while considering the entire genome of the organism, the predicted strategies can sometimes prove to be difficult for experimental validation. Therefore, to predict the optimal strategy for production of any metabolite with complete accuracy, it is desirable to have a detailed genome-scale dynamic model of metabolism, which is currently impractical because of the lack of information on kinetic parameters and regulatory network. Under this scenario, the modeling procedure discussed in this work provides an effective way to make a genome-scale experimental design.

5. ACKNOWLEDGEMENTS

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