**An Updated Approach to Condition-Specific Metabolic Modeling**

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**Abstract**

**Background**

The integration of experimental data to predict metabolic changes in genome scale metabolic models is an active field of research in systems biology. It is imperative to develop software that can test condition-specific modeling across a wide range of organisms and data types.

**Results**

We have developed EXploration of Alternative Metabolic Optima – Adapted for Robustness, Conversion, and Visualization: 1 (EXAMO-ARC.V.1). It can predict the metabolic fluxes for a condition given a generic set of gene activity rules mapped to proteins and metabolic reactions. Many advancements were made to the original software implementation. These include the preservation of Boolean gene rules for reaction activity, the randomization of attempting to reduce the complexity of the model, and enhancing the robustness of minimizing the model without failing. In addition, a model importing script was created, allowing for the import of metabolic reconstruction files. If desired, models can also be adapted to reduce cofactors and nucleoside phosphate moieties to enhance carbon-centered predicted fluxes. Visualization tools were established to aid in contextualizing metabolic effects for specific pathways. The software was also adapted so that it could be run on any Linux distribution. Publically available transcriptomic datasets were used to generate gene rules and validate the software. Gene-deletion mutant studies in *Saccharomyces cerevisiae* grown in glucose or ethanol were used to determine the precision and sensitivity of reactions included in the reduced models. Errors in predicting fluxes were determined based off of metabolic flux analysis generated fluxes from aerobic and anaerobic conditions. The updated software outperformed the original implementation in reaction inclusivity and predicted fluxes.

**Conclusions**

We have designed software that can import the metabolic reconstruction of any model organism and predict and visualize fluxes for a condition based on predefined gene rules. The model building algorithm reduces models relatively quickly for small model organisms, making calculations feasible on a local computer. Predicting condition-specific *in silico* fluxes has widespread applications ranging from engineering bacterial and yeast strains for biomass production, to predicting tissue-specific models, to characterizing general metabolic phenotypes for an experiment. EXAMO-ARC.V.1 has the potential to be utilized by a wide spectrum of researchers.

**Keywords**: EXAMO, FBA, iMAT, MBA, flux, condition-specific, environment-specific, tissue-specific, metabolic, modeling

**Background**

Flux balance analysis (FBA), or the *in silico* modeling of the flux of nutrients through a biological system using linear programming, can be used to model metabolism. Several mathematical algorithms have been developed to predict fluxes using FBA for tissue-specific or condition-specific constraint-based models [1–4]. Many of these algorithms differ in what they are trying to optimize, with some incorporating gene rules or discretized expression of genes directly into the optimization. Others do not solve for a flux state, but rather just predict a set of core reactions that are important for metabolic function [5–7]. Some require multiple data sets or time course experiments to predict flux states [7–9]. EXploration of Alternative Metabolic Optima (EXAMO), developed by Rossell et al, is the most computationally expensive of these algorithms. It uses a heuristic model-building algorithm (MBA), which tries to remove one reaction at a time from the reactions in the model while not removing a core set of reactions defined to be active based off of gene rules, to create a reduced functional model representative of the condition of interest [10]. One of the benefits of the algorithm, however, is that it can create phenotypically predictive models using only one data set [1,9]. This is due to its flexibility of utilizing various types of high throughput data to generate gene rules, such as by means of gene expression thresholds or statistical differences between conditions, or a combination of the two. It can glean phenotypic metabolic function by optimizing for an objective function of biomass metabolites, or in other terms, the metabolic goal of the system. EXAMO creates an alternative version of this optimization by minimizing the sum of fluxes in the system with a defined nonzero lower boundary biomass production to minimize metabolic feedback loops in the system. The authors were able to predict the Crabtree effect, whereby glucose induction produces aerobic glycolysis and alcoholic fermentation [10,11]. However, it was difficult to use the algorithm across platforms, to use other metabolic reconstructions, and the algorithm did not always succeed in generating a flux result with a given gene rule set.

**Implementation**

The EXAMO software was adapted to be more user-friendly and robust; the new implementation is called Exploration of Alternative Metabolic Optima – Adapted for Randomization and Compatibility (EXAMO-ARC.V.1). Previously, the algorithm had been comprised of four Python scripts. The first script accepted a set of gene rules, and it classified reactions as being highly (rH) or lowly (rL) expressed based off the gene-protein-reaction (GPR) associations. It used a mixed integer linear program using the methodology from the Integrated Metabolic Analysis Tool (iMAT) to find the optimal reaction classification system, whereby the most rH reactions and the fewest rL reactions had a flux [5,12]. What this equated to was a maximization of pathway length to agree with the reaction rules, so that the most favorable path was found to achieve biomass production (when the biomass reaction had a nonzero lower boundary constraint). Reactions always or never included in the set of most optimal classification solutions were classified as high frequency reactions (HFRs) or zero frequency reactions (ZFRs), respectively. These reactions were then used as the basis for inclusion or exclusion in the reduced condition-specific models using the MBA [13]. ZFRs were removed first from consideration. HFRs were preserved and were not able to be pruned from the model. Reactions were heuristically removed one-by-one from the model unless a pruned reaction forced any of the HFRs to be unable to produce a flux. Any resulting reactions that became inactive along a pathway due to the removal of a pruned reaction were also removed themselves. A profile of pruned models was thereby created for a user-defined number of repetitions the MBA was to be completed. The third script added reactions one-by-one back to the set of HFRs in the order of the frequency of the reactions in the model profile, until all of the HFRs were able to produce a flux [13]. The fourth script then found a steady-state flux solution for a biomass producing system by minimizing the sum of fluxes as the objective function to minimize metabolic feedback loops in the system [10]. In the new implementation, the second through fourth scripts are adapted into one to ensure robustness of minimizing the model and being able to determine a nonzero biomass flux solution without failing. Every generated pruned model in the profile is now solvable itself. Other adaptations to these scripts included preserving GPR parsing to account for AND and OR logic gates for proteins in complex or if there are isoforms or separate complexes available for a reaction. The order of pruning reactions in the MBA was also randomized using the Python Cryptography Toolkit, so that not every repetition would produce the same results [14].

EXAMO-ARC.V.1 also contains model importing, model adaptation, and flux visualization capabilities. A script was generated to import systems biology markup language (SBML) models and COnstraint Based Reconstruction Analsyis (COBRA) models in .xml or .mat formats, respectively, and recreate them in the necessary Python pickle format (.pkl) that EXAMO uses [15,16]. The importing script also contains the capability of simplifying the model in several different ways if the user desires, such as by only including carbon balanced reactions, removing metabolites without carbons, and converting all nucleoside phosphate moieties and cofactor moieties into common shared metabolites. These simplifications would help to focus the modeling on carbon driven reactions. The importing script also exports the model as a .mat COBRA file for the capability of modeling in other programs. In the MBA, there is now also the user-defined option to choose in what order to prune reactions. By first pruning extracellular reactions, then extracellular transport reactions, then compartmental transport reactions, and then attempting to remove inner-compartmental reactions, more of an emphasis is placed on the importance of transport reaction gene activity for defining the metabolic state of the system. A description of all testing parameters for EXAMO-ARC.V.1 from user-defined options is described in **Table 1**. A list of the GPR associations changed for extracellular metabolite uptake is described in **Supplementary Table 1**, in accordance with literature updates. A description of the lower boundary constraint changes for extracellular metabolite uptake for each condition tested is described in **Supplementary Table 2**. The cofactor and nucleoside phosphate moiety conversions are described in **Supplementary Table 3**. A visualization scripts was also created to map fluxes onto user-generated metabolic pathways designed in CellDesigner [17]. Lastly, EXAMO-ARC.V.1 is now operable on more Linux systems, as the \_\_init\_\_.py script has been included to correctly reference modules and recreate the serialized .pyc file for a user’s own system. A general outline of the algorithm is shown in **Figure 1**.

**Table 1**

|  |  |  |  |
| --- | --- | --- | --- |
| **Condition** | **Script** | **Function** | **Logic** |
| g | conversitonScript.py | Change the GPR association | Account for additional gene associations from literature |
| lb | conversionScript.py | Adjust the lower boundary constraints | Adjust model to account for extracellular media/uptake rates |
| m\_n\_c | conversionScript.py | Account for cofactors and nucleoside phosphate moieties, and make the model carbon balanced | Make flux model more carbon-centered |
| Ex | examo\_2\_4.py | Prune extracellular reactions first, then extracellular transport reactions, then compartmental transport reactions, then inner-compartmental transport reactions | More of an emphasis is placed on transport reaction gene activity |

A description of the testing parameters used for EXAMO-ARC.V.1, as well as the script in which the optional parameter is available, the function of the parameter, and the logic behind the change.

**Figure 1**

**EXAMO-ARC.V.1: The process of building a functional model and solving for a flux state**

First the metabolic reconstruction (SBML or .mat file) is converted to the necessary Python objects (as a pickle file). Next, discretized data is used for gene rules to determine which reactions are predicted to be turned on/off based on the gene to protein to reaction mappings. Next, reaction activity is determined in accordance with the gene rules to determine high frequency reactions (HFRs) and zero frequency reactions (ZFRs), based on flux variability analysis (FVA). Then, a model building algorithm (MBA) is run repeatedly to remove all ZFRs and then undefined reactions one-by-one heuristically to create a profile of reduced models (reaction inclusion consistency). The undefined reactions are then added to the HFRs in the order of their frequencies in the profile until biomass can be achieved. A minimization of the sum of fluxes is determined with a minimum biomass constraint imposed, and the fluxes are visualized. The pipeline compartments highlight the origins of the used programs, algorithms, and concepts.

To test how well the model building algorithm in EXAMO-ARC.V.1 removed reactions from the genome scale model to create biologically meaningful condition-specific models, gene rules were defined from transcriptomic datasets from Gasch et al for *Saccharomyces cerevisiae* cultures grown in glucose and ethanol and compared to essential genes based off of gene-deletion mutant studies by Giaever et al and Snitkin et al in glucose and ethanol, respectively [18–20]. In brief, the microarray datasets were downloaded using GEOquery and the probes were normalized across genes using affyPLM in R [21,22]. The iMM904 *Saccharomyces cerevisiae* metabolic reconstruction was used in the simulations, with the adaptations made for NAD biosynthesis [23,24]. The reaction for formate-tetrahydrate ligase was made to be irreversible, as represented in the recent human metabolic reconstruction [25]. For the default constraints for extracellular media uptake, the lower boundaries were left open by default, as in the original EXAMO study [10]. The genes in the iMM904 model with expression in the highest and lowest 25% of genes were classified as active and inactive, respectively, following similar expression thresholds as in other studies [2,6]. By default, the lower boundary constraints were left open, but the detailed concentrations of YPD or YPEtoh (glucose replaced by ethanol) with the calculated amino acid, vitamin, and nucleotide compositions in the yeast extract were used as alternative lower boundaries [26–28]. After running the models with the parameters previously described for EXAMO-ARC.V.1 and EXAMO (when possible), sensitivity (***tp/(tp+fn)***) and precision (***tp/(tp+fp)***) were calculated based off of the reduced models for the included genes that had been tested experimentally for each condition. For the gene-deletion mutant studies, essential genes were classified as homozygous mutants producing slow to no growth. True positives (*tp*) refer to included reactions that were deemed essential, false positives (fp) were genes that were deemed essential but were not experimentally, and false negatives (fn) were genes that were removed from the model but were actually essential. Similar to other studies, genes essential in YPD were not considered for testing when considering essential genes for YPEtoh, as they may be essential under all conditions [10,20].

To analyze the accuracy of EXAMO-ARC.V.1 predicted fluxes, fluxes calculated from aerobic and anaerobic transcriptomic datasets were compared to fluxes calculated from metabolic flux analysis experiments in the same conditions. Gene rules were defined from microarray experiments from Rintala et al at 20.9% and 0% oxygen concentrations with glucose in the media [29]. The microarray datasets were downloaded using GEOquery in R, and the probes across replicates and genes were averaged [21]. The 25% expression threshold was again used to define gene activity. The detailed concentrations of components in the media were used for determining the lower boundary constraints for extracellular metabolite uptake as an optional parameter when formatting the model [30]. Fluxes from EXAMO and EXAMO-ARC.V.1 were compared to fluxes determined by METAFoR for a simplified metabolic network using 13C-labeled 2D NMR tracer experiments for the same strains in the same conditions as in Rintala et al’s study [29,31,32]. The differences in fluxes were calculated (***experimentally determined – simulated***) for the overlapping reactions in both the iMM904 model and the simplified model.

Need to include note about lower boundary for biomass for original EXAMO software (not 100 but 0.2879) and about eps.

Need to note that for multiple gene mapping gates, if any produced a 1 or 0, this was preferred over something that produced a -1 (more conservative).

**Results**

**Figure 2**

**Comparison of Sensitivity, Precision, and Solvability of Conditions and Software**

Sensitivity (**a**) and precision (**b**) of reaction inclusion based off of experimental gene knockout data was analyzed for the condition-specific models generated from the gene rules for ethanol, glucose, and their respective negative controls. The different implementations of the EXAMO software were also compared. For the original EXAMO software and original model included in the distribution shown in light blue and for the original EXAMO software using the same model that was tested in EXAMO-ARC.V.1 shown in light orange, epsilon was changed to 1E-10 when solving the reduced network (C\_orig\_eps and C\_mod\_eps, respectively). Shown in light red, EXAMO-ARC.V.1 was tested using combinations of model parameters as described in Table 1. If all repetitions of a condition for a parameter were not solvable, the sensitivity and precision were 0. The sensitivities and precisions of the parameters of EXAMO-ARC.V.1 were best when cofactors, nucleoside phosphate moieties, and carbon balancing (m\_n\_c) as well as lower boundary constraints (lb) were not imposed, producing results that were better than the negative controls. The sensitivities of these particular parameters were better than when the original EXAMO software was used, but the precisions were not better. (**c**)A comparison of the solvability of pruned models and optimizations. Five repetitions of 50 iterations are stacked on top of each other. All EXAMO-ARC.V.1 models were able to be pruned and were solvable. In contrast, most models of the original implementation of EXAMO were able to be pruned, but not all repetitions of EXAMO were solvable.

**Figure 3**

**Comparison of Accuracy of Flux Profiles**

Simulated fluxes from conditions-specific models for aerobic and anaerobic gene rules and their negative controls were compared to experimentally modeled flux results using modeled 2D NMR tracer experimental results under the same aerobic and anaerobic conditions. The absolute flux difference was compared and plotted by metabolic pathway for the overlapping reactions between the experimental and simulated results. If a reaction corresponded to more than one reaction in the larger metabolic reconstruction, the redundant reactions are noted, and the fluxes were added together for these reactions. The total absolute flux difference of all reactions for a parameter for a condition is noted at the top of each column of the heatmaps. The aerobic condition with cofactors, nucleoside phosphate moieties, and carbon balancing (m\_n\_c) produced the most accurate flux results of the experimental fluxes compared to the simulated fluxes; the negative control had a greater difference as well compared to the modeled gene rules for the aerobic condition.

**Figure 4**

**Comparison of Flux Predictions**

Flux differences were compared for the aerobic and anaerobic conditions versus the negative control for each simulation platform (**a**) and across platforms (**b**). For EXAMO-ARC.V.1, the model parameters with cofactors, nucleoside phosphate moieties, and carbon balancing (m\_n\_c) were combined to make a C\_m\_n\_c Cohort, and all model parameters excluding m\_n\_c were also combined to make a C\_non\_m\_n\_c Cohort. For (**a**), a two-way ANOVA with replicates was performed on the total absolute flux difference of the five replicates for each model parameter, comparing the negative control versus each condition for a software platform and accounting for each parameter group for the cohorts of EXAMO-ARC.V.1, shown in light red. A one-way ANOVA with replicates was used for testing the difference between the negative control versus each condition for the original EXAMO software with the model converted by EXAMO-ARC.V.1, shown in light yellow. The modeled gene rules for both the aerobic and anaerobic conditions had significantly more accurate results than the negative controls for the original EXAMO distribution with the model converted by EXAMO-ARC.V.1 and for EXAMO-ARC.V.1 for the C\_m\_n\_c Cohort. For (**b**), a two-way ANOVA with replicates was used comparing the total absolute flux difference of the original software with the two cohorts for EXAMO-ARC.V.1 while accounting for the model parameter groups. EXAMO-ARC.V.1 produced more accurate results for both of the cohorts. (**c**) A representation of fluxes per reaction per pathways of interest for the aerobic and anaerobic conditions for the C\_m\_n\_c\_lb\_g parameter, which produced the lowest combined absolute flux difference between the experimental and simulated results.

**Figure 5**

**Flux Maps of Central Carbon Metabolism for the Aerobic and Anaerobic Conditions**

Flux maps were designed in CellDesigner and overlaid with flux profiles for the (**a**) Aerobic and (**b**) Anaerobic conditions for the C\_m\_n\_c\_lb\_g parameter using mapCreate.py from EXAMO-ARC.V.1. As expected, the anaerobic condition had a greater glycolytic flux. It also had a greater flux through the pentose phosphate pathway and serine/glycine metabolism. In comparison, the aerobic condition utilized branching off of glycolysis through lactate, and it was also predicted to produce alanine biosynthetically from pyruvate. Reactions with negative fluxes, such as PGK and PGM, have been adjusted through mapCreate.py to flip the reactions from the way they were originally encoded. Their flux annotations are preceded by a double underscore. If a flux or its standard deviation are followed by e\_4, such as the flux 121109e\_4 for ENO for the anaerobic condition, this translates to 12.1109, whereas e\_4 is scientific notation for E-4.

**Discussion**

**Conclusion**

* EXAMO-ARC V:1 is more robust than EXAMO.
* EXAMO-ARC V:1 now allows for importing of any model, adaptations to models, and visualization of fluxes.
* Reducing the model complexity in terms of cofactors and nucleoside phosphate moieties produces models closer to the actual fluxes.
* Gene thresholding of expression does not do a good job at predicting reaction inclusion compared to null gene rules.
* Need to analyze the results more to compare further differences between EXAMO-ARC V:1 and EXAMO.

**List of Abbreviations**

**Declarations**

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Availability of data and material**

**Competing interests**

The authors declare that they have no competing interests.

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