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INTRODUCTION:

In this tutorial we will run optForce. For a detailed description of the procedure, please see [1]. Briefly, the problem is to find a set of interventions of size "K" such that when these interventions are applied to a wild-type strain, the mutant created will produce a particular target of interest in a higher rate than the wild-type strain. The interventions could be knockouts (lead to zero the flux for a particular reaction), upregulations (increase the flux for a particular reaction) and downregulations (decrease the flux for a particular reaction).

For example, imagine that we would like to increase the production of succinate in Escherichia coli. Which are the interventions needed to increase the production of succinate? We will approach this problem in this tutorial and we will see how each of the steps of OptForce are solved.

MATERIALS

EQUIPMENT

1. MATLAB
2. GAMS
3. A solver for Mixed Integer Linear Programming (MILP) problems. For example, Gurobi.

EQUIPMENT SETUP

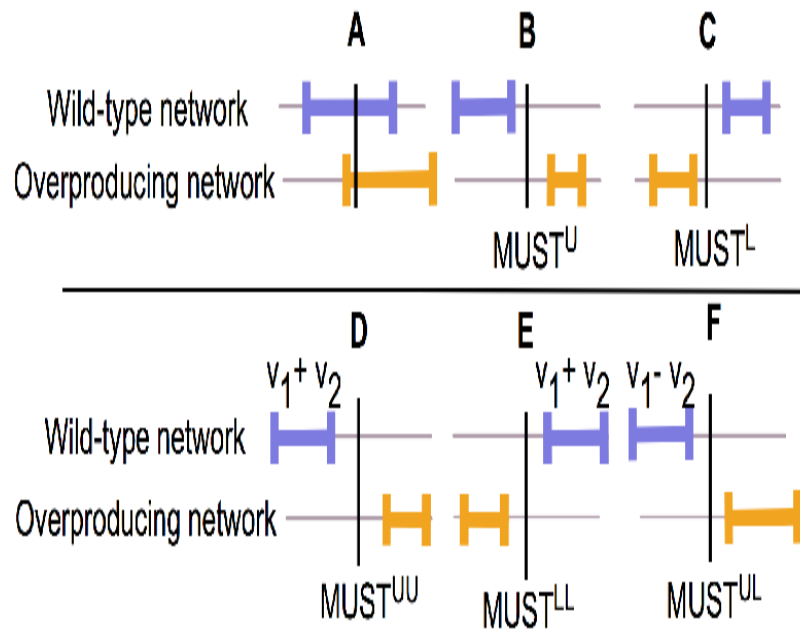
GAMS: Install the latest version of GAMS available. This is important as some GAMS functions are not supported in versions older than 24.7.1. During GAMS installation make sure to select the option "Add GAMS directory to PATH environment variable". In MATLAB, use functions `addpath()` and `savepath()` for adding and saving the directory where GAMS was installed. For a more detailed description of GAMS installation and setup, you can watch the official tutorial of GDXMRW at https://www.youtube.com/watch?v=HPn_q8nlktE

PROCEDURE

The procedure consists on the following steps

- 1) Maximize specific growth rate and product formation.
- 2) Define constraints for both wild-type and mutant strain:
- 3) Perform flux variability analysis for both wild-type and mutant strain.
- 4) Find must sets, i.e, reactions that MUST increase or decrease their flux in order to achieve the phenotype in the mutant strain.

Figure 1.



5) Find the interventions needed that will ensure a increased production of the target of interest

Now, we will approach each step in detail.

STEP 1: Maximize specific growth rate and product formation

First, we load the model. This model comprises only 90 reactions, which describe the central metabolism of *E. coli* [2].

Then, we change the objective function to maximize biomass ("R75"). We also change the lower bounds, so *E. coli* will be able to consume glucose, oxygen, sulfate, ammomium, citrate and glycerol.

```
global TUTORIAL_INIT_CB;
if ~isempty(TUTORIAL_INIT_CB) && TUTORIAL_INIT_CB==1
    initCobraToolbox(false) % false, as we don't want to update
    changeCobraSolver('gurobi','all');
end

pathTutorial = which('tutorial_OptForceGAMS.mlx');
pathstr = fileparts(pathTutorial);
cd(pathstr)

modelName = 'AntCore.mat';
modelDirectory = getDistributedModelFolder(modelName); %Look up the folder for the
modelName = [modelDirectory filesep modelName]; % Get the full path. Necessary t
model = readCbModel(modelName);

model.c(strcmp(model.rxns,'R75')) = 1;
model = changeRxnBounds(model, 'EX_gluc', -100, '1');
model = changeRxnBounds(model, 'EX_o2', -100, '1');
model = changeRxnBounds(model, 'EX_so4', -100, '1');
```

```

model = changeRxnBounds(model, 'EX_nh3', -100, 'l');
model = changeRxnBounds(model, 'EX_cit', -100, 'l');
model = changeRxnBounds(model, 'EX_glyc', -100, 'l');

```

Then, we calculate the maximum specific growth rate and the maximum production rate for succinate

```

growthRate = optimizeCbModel(model);
fprintf('The maximum growth rate is %1.2f', growthRate.f);

```

The maximum growth rate is 14.36

```

model = changeObjective(model, 'EX_suc');
maxSucc = optimizeCbModel(model);
fprintf('The maximum production rate of succinate is %1.2f', maxSucc.f);

```

The maximum production rate of succinate is 155.56

TIP: The biomass reaction is usually set to 1%-10% of maximum theoretical biomass yield when running the following steps, to prevent solutions with not biomass formation

1. maximizing product formation
2. finding MUST sets of second order
3. finding FORCE sets

STEP 2: Define constraints for both wild-type and mutant strain

TIMING: This step should take a few days or weeks, depending on the information available for your species.

CRITICAL STEP: This is a manual task, so you should search for information in articles or even perform your own experiments. You can also make assumptions for describing the phenotypes of both strains which will make this task a little faster but make sure to have two strains different enough, because you should be able to find differences in reactions ranges.

First, we load the model. This model comprises only 90 reactions, which describe the central metabolism of E. coli [2].

Then, we change the objective function to maximize biomass ("R75"). We also change the lower bounds, so E. coli will be able to consume glucose, oxygen, sulfate, ammomium, citrate and glycerol.

We define constraints for each strain

```

Constr_WT = struct('rxnList', {'R75'}, 'rxnValues', 14, 'rxnBoundType', 'b');
Constr_MT = struct('rxnList', {'R75', 'EX_suc'}, 'rxnValues', [0, 155.55], 'rxnBoundType', 'b');

```

Step 3: Flux Variability Analysis

TIMING: This task should take from a few seconds to a few hours depending on the size of your reconstruction

We run the FVA analysis for both strains

```

[minFluxes_WT, maxFluxes_WT, minFluxes_MT, maxFluxes_MT,~,~] = FVAOptForce(model, Constr_WT, Constr_MT);

```

```
disp([minFluxes_WT, maxFluxes_WT, minFluxes_MT, maxFluxes_MT]);
```

-90.1251	97.1300	44.4313	100.0000
0	86.0700	44.4375	100.0000
0	86.0700	44.4375	100.0000
-56.1567	86.0700	-44.4500	11.1143
21.3033	163.5300	55.5500	111.1143
-3.0777	154.8640	55.5500	111.1143
0	151.5086	0	55.5625
0	187.2551	0	55.5687
0	169.5163	0	0.0187
-10.0660	102.9449	0	0.0125
10.0660	66.5714	0	0.0063
-10.0660	102.9449	0	0.0125
-48.9454	7.5600	-0.0063	0
-53.9994	2.5060	-0.0063	0
-53.9994	2.5060	-0.0063	0
-2.5060	53.9994	0	0.0063
0	86.0700	0	55.5625
0	86.0700	0	55.5625
9.7020	114.6466	55.5500	55.5625
0	56.5564	55.5500	55.5571
16.0264	145.2048	155.5500	155.5563
16.0264	145.2048	155.5500	155.5563
0.9344	130.1128	155.5500	155.5562
-5.6736	123.5048	155.5500	155.5563
0	118.0576	0	0.0062
5.1940	123.2516	0	0.0062
-98.1150	123.2516	-55.5625	0.0062
0	151.5086	0	55.5625
0	151.5086	0	55.5625
0	254.5400	55.5500	777.7875
0	253.2493	0	722.2375
-7.1960	94.6056	0	0.0125
0	84.8467	88.8750	88.9000
0	84.8467	88.8750	88.9000
0	175.1064	188.8500	188.9000
0	175.1064	188.8500	188.9000
91.4130	107.1280	0	0
9.4500	9.4500	0	0
2.9400	2.9400	0	0
3.9340	3.9340	0	0
25.4520	56.8820	0	0
3.2060	3.2060	0	0
6.8320	6.8320	0	0
0	15.7150	0	0
-6.8880	8.8270	0	0
0.6790	16.3940	0	0
0	31.4300	0	0
3.2620	3.2620	0	0
4.5640	4.5640	0	0
4.5640	4.5640	0	0
7.2380	38.6680	0	0
2.0440	2.0440	0	0
5.6280	5.6280	0	0
5.9920	5.9920	0	0
3.8640	3.8640	0	0
2.4640	2.4640	0	0
1.8340	1.8340	0	0
0.7560	0.7560	0	0
1.2600	1.2600	0	0
2.0440	2.0440	0	0
1.2600	1.2600	0	0
79.7324	200.0000	199.9500	200.0000

0	118.0576	0	0.0062
-39.5563	353.9124	-22.2500	33.3500
0	253.2493	0	722.2375
40.6268	100.0000	99.9875	100.0000
15.0890	100.0000	99.9929	100.0000
-100.0000	84.8467	-100.0000	-99.9500
0	175.1064	188.8500	188.9000
0	101.8016	0	0.0125
134.9718	407.3274	311.1000	311.1187
62.1267	100.0000	99.9750	100.0000
97.4820	97.4820	0	0
3.2620	3.2620	0	0
14.0000	14.0000	0	0
0	175.1064	188.8500	188.9000
134.9718	407.3274	311.1000	311.1187
0	101.8016	0	0.0125
0	253.2493	0	722.2375
-100.0000	-40.6268	-100.0000	-99.9875
-100.0000	-15.0890	-100.0000	-99.9929
-100.0000	84.8467	-100.0000	-99.9500
-97.4820	-97.4820	0	0
-100.0000	-62.1267	-100.0000	-99.9750
-3.2620	-3.2620	0	0
0	105.4230	155.5500	155.5500
0	105.4230	155.5500	155.5500
11.6200	11.6200	0	0
5.0540	5.0540	0	0
5.9920	5.9920	0	0

Now, the run the second step of OptForce.

Step 4: Find Must Sets

TIMING: This task should take from a few seconds to a few hours depending on the size of your reconstruction

First, we define an ID for this run. Each time you run the functions associated to the optForce procedure, some folders can be generated to store inputs used in that run. Outputs are stored as well. These folder will be located inside the folder defined by your run ID. Thus, if your runID is "TestOptForce", the structure of the folders will be the following:

CurrentFolder

```
|   TestOptForce
|   |   Inputs
|   |   Outputs
```

To avoid the generation of inputs and outputs folders, set keepInputs = 0, printExcel = 0, printText = 0 and keepGamsOutputs = 0

Also, a report of the run is generated each time you run the functions associated to the optForce procedure. So, the idea is to give a different runID each time you run the functions, so you will be able to see the report (inputs used, outputs generated, errors in the run) for each run.

We define then our runID

```
runID = 'TestOptForce' ;
```

Now, only functions to find first and second order must sets are supported. As depicted in **Figure 1**, the first order must sets are MUSTU and MUSTL; and second order must sets are MUSTUU, MUSTLL and MUSTUL

A) Finding first order must sets

We define constraints

```
constrOpt = struct('rxnList', {{'EX_gluc','R75','EX_suc'}}, 'values', [-100, 0, 155.5])
```

We then run the functions findMustLWithGAMS.m and findMustUWithGAMS.m that will find mustU and mustL sets, respectively.

Important: To run these function you will need a solver able to solve Mixed Integer Linear Programming (MILP or MIP) problems. Some popular options are: cplex and gurobi. You can see which gams solvers are available in your systems to solve MIP problems by running checkGAMSSolvers('MIP')

```
solvers = checkGAMSSolvers('MIP'); disp(solvers);
```

Columns 1 through 12

```
'AMPL' 'BARON' 'BDMLP' 'BENCH' 'CBC' 'CONVERT' 'CPLEX' 'EXAMINER' 'GAMSCHK'
```

Columns 13 through 22

```
'LINDOGLOBAL' 'LINGO' 'LOCALSOLVER' 'MOSEK' 'MPECDUMP' 'OsiCplex' 'OsiGurobi' 'Os
```

Columns 23 through 25

```
'SCIP' 'XA' 'XPRESS'
```

We then run findMustLWithGAMS.m and findMustUWithGAMS.m.

i) MustL Set:

```
[mustLSet, pos_mustL] = findMustLWithGAMS(model, minFluxes_WT, maxFluxes_WT, 'constrOpt', 'solverName', 'cplex', 'runID', runID, 'outputFolder', 'OutputsFindMustL', 'outputFileName', 'printExcel', 1, 'printText', 1, 'printReport', 1, 'keepInputs', 1, 'keepGamsOutputs', 1);
```

Note that the folder "TestOptForce" was created. Inside this folder, two additional folders were created: "InputsMustL" and "OutputsFindMustL". In the inputs folder you will find all the inputs required to run the GAMS function "findMustL.gms". Additionally, in the outputs folder you will find the mustL set found, which were saved in two files (.xls and .txt), as well as other files generated automatically by GAMS. Furthermore, a report which summarize all the inputs and outputs used during your running was generated. The name of the report will be in this format "report-Day-Month-Year-Hour-Minutes". So, you can maintain a chronological order of your experiments

We display the reactions that belongs to the mustL set

```
disp(mustLSet)
```

```
'R11'  
'R26'  
'R37'  
'R38'
```

```
'R39'
'R40'
'R41'
'R42'
'R43'
'R46'
'R48'
'R49'
'R50'
'R51'
'R52'
'R53'
'R54'
'R55'
'R56'
'R57'
'R58'
'R59'
'R60'
'R61'
'R73'
'R74'
'PSEUDOpYr_1'
'PSEUDOpEp_1'
'PSEUDOCO2_1'
```

ii) MustU set:

```
[mustUSet, pos_mustU] = findMustUWithGAMS(model, minFluxes_WT, maxFluxes_WT, 'constrOpt',
'solverName', 'cplex', 'runID', runID, 'outputFolder', 'OutputsFindMustU', 'outputFileM',
'printExcel', 1, 'printText', 1, 'printReport', 1, 'keepInputs', 1, 'keepGamsOutputs',
```

Note that the folders "InputsMustU" and "OutputsFindMustU" were created. These folders contain the inputs and outputs of findMustUWithGAMS.m, respectively.

We display the reactions that belongs to the mustU set

```
disp(mustUSet)
```

```
'R21'
'R22'
'R23'
'R24'
'R33'
'R34'
'R35'
'R36'
'R69'
'EX_pdo'
'EX_nh3'
'EX_so4'
'SUCt'
```

B) Finding second order must sets

First, we define the reactions that will be excluded from the analysis. It is suggested to eliminate reactions found in the previous step as well as exchange reactions

```

constrOpt = struct('rxnList', {{'EX_gluc','R75','EX_suc'}},'values', [-100, 0, 155.5]);
exchangeRxns = model.rxns(cellfun(@isempty, strfind(model.rxns, 'EX_')) == 0);
excludedRxns = unique([mustUSet; mustLSet; exchangeRxns]);
mustSetFirstOrder = unique([mustUSet; mustLSet]);

```

Now, we run the functions for finding second order must sets

i) MustUU:

```

[mustUU, pos_mustUU, mustUU_linear, pos_mustUU_linear] = findMustUUWithGAMS(model, minE,
    'constrOpt', constrOpt, 'excludedRxns', excludedRxns, 'mustSetFirstOrder', mustSetFirstOrder,
    'runID', runID, 'outputFolder', 'OutputsFindMustUU', 'outputFileName', 'MustUU', 'printReport',
    'printReport', 1, 'keepInputs', 1, 'keepGamsOutputs', 1, 'verbose', 0);

```

Note that the folders "InputsMustUU" and "OutputsFindMustUU" were created. These folders contain the inputs and outputs of findMustUUWithGAMS.m, respectively.

We display the reactions that belongs to the mustUU set

```
disp(mustUU);
```

```

'R30'      'R65'
'R65'      'R31'

```

ii) MustLL:

```

[mustLL, pos_mustLL, mustLL_linear, pos_mustLL_linear] = findMustLLWithGAMS(model, minE,
    'constrOpt', constrOpt, 'excludedRxns', excludedRxns, 'mustSetFirstOrder', mustSetFirstOrder,
    'runID', runID, 'outputFolder', 'OutputsFindMustLL', 'outputFileName', 'MustLL', 'printReport',
    'printReport', 1, 'keepInputs', 1, 'keepGamsOutputs', 1, 'verbose', 0);

```

Note that the folders "InputsMustLL" and "OutputsFindMustLL" were created. These folders contain the inputs and outputs of findMustLLWithGAMS.m, respectively.

We display the reactions that belongs to the mustLL set. In this case, MustLL is an empty array because no reaction was found in the mustLL set.

```
disp(mustLL);
```

iii) MustUL:

```

[mustUL, pos_mustUL, mustUL_linear, pos_mustUL_linear] = findMustULWithGAMS(model, minE,
    'constrOpt', constrOpt, 'excludedRxns', excludedRxns, 'mustSetFirstOrder', mustSetFirstOrder,
    'runID', runID, 'outputFolder', 'OutputsFindMustUL', 'outputFileName', 'MustUL', 'printReport',
    'printReport', 1, 'keepInputs', 1, 'keepGamsOutputs', 1, 'verbose', 0);

```

Note that the folders "InputsMustUL" and "OutputsFindMustUL" were created. These folders contain the inputs and outputs of findMustULWithGAMS.m, respectively.

We display the reactions that belongs to the mustUL set. In this case, MustUL is an empty array because no reaction was found in the mustUL set.


```
disp(mustUL);
```

TROUBLESHOOTING 1: "I didn't find any reaction in my must sets"

TROUBLESHOOTING 2: "I got an error when running the findMustXWithGAMS.m functions (X = L or U or LL or UL or UU depending on the case)"

Step 5: OptForce

TIMING: This task should take from a few seconds to a few hours depending on the size of your reconstruction

We define constraints and we define "K" the number of interventions allowed, "nSets" the maximum number of sets to find, and "targetRxn" the reaction producing the metabolite of interest (in this case, succinate).

Additionally, we define the mustU set as the union of the reactions that must be upregulated in both first and second order must sets; and mustL set as the union of the reactions that must be downregulated in both first and second order must sets

```
mustU = unique(union(mustUSet, mustUU));
mustL = unique(union(mustLSet, mustLL));
targetRxn = 'EX_suc';
k = 1;
nSets = 1;
constrOpt = struct('rxnList', {{'EX_gluc', 'R75'}}, 'values', [-100, 0]);

[optForceSets, posOptForceSets, typeRegOptForceSets] = optForceWithGAMS(model, targetRxn,
    maxFluxes_WT, minFluxes_MT, maxFluxes_MT, 'k', k, 'nSets', nSets, 'constrOpt', constrOpt,
    'runID', runID, 'outputFolder', 'OutputsOptForce', 'outputFileName', 'OptForce', 's',
    'printReport', 1, 'keepInputs', 1, 'keepGamsOutputs', 1, 'verbose', 0);
```

Note that the folders "InputsOptForce" and "OutputsOptForce" were created. These folders contain the inputs and outputs of optForceWithGAMS.m, respectively.

We display the reactions found by optForce

```
disp(optForceSets)
```

```
'SUCt'
```

The reaction found was "SUCt", i.e. a transporter for succinate (a very intuitive solution).

Next, we will increase "k" and we will exclude "SUCt" from upregulations to found non-intuitive solutions.

TIP: Sometimes the product is at the end of a long linear pathway. In that case, the recommendation is to also exclude most reactions on the linear pathway. Essential reactions and reactions not associated with any gene should also be excluded.

We will only search for the 20 best solutions, but you can try with a higher number.

We will change the runID to save both results (k = 1 and K = 2) in different folders

```

k = 2;
nSets = 20;
runID = 'TestOptForce2';
excludedRxns = struct('rxnList', {{'SUCt'}} , 'typeReg','U');
[optForceSets, posOptForceSets, typeRegOptForceSets] = optForceWithGAMS(model, targetRxns,
    maxFluxes_WT, minFluxes_MT, maxFluxes_MT, 'k', k, 'nSets', nSets, 'constrOpt', constraints,
    'runID', runID, 'outputFolder', 'OutputsOptForce', 'outputFileName', 'OptForce', 'suffix',
    'printReport', 1, 'keepInputs', 1, 'keepGamsOutputs', 1, 'verbose', 0);

```

Note that the folders "InputsOptForce" and "OutputsOptForce" were created inside TestOptForce2. These folders contain the inputs and outputs of optForceWithGAMS.m, respectively.

We display the reactions found by optForce

```
disp(optForceSets)
```

```

'R23'    'R26'
'R24'    'R25'
'R24'    'R26'
'R23'    'R25'
'R23'    'R26'
'R23'    'R63'
'R22'    'R25'
'R24'    'R63'
'R21'    'R26'
'R24'    'R26'
'R22'    'R26'
'R22'    'R63'
'R22'    'R26'
'R21'    'R63'
'R21'    'R25'
'R21'    'R26'
'R24'    'R4'
'R23'    'R4'
'R22'    'R4'
'R21'    'R4'

```

TIMING

1. STEP 1: < 1 second
2. STEP 2: ~ 1-2 seconds
3. STEP 3: ~ 2-5 seconds
4. STEP 4: ~ 10-20 seconds
5. STEP 5: ~ 10-20 seconds

TROUBLESHOOTING

1) problem: "I didn't find any reaction in my must sets"

possible reason: the wild-type or mutant strain is not constrained enough.

solution: add more constraints to your strains until you find differences in your reaction ranges. If you don't find any differences, it is better to change the approach and use another algorithm. Also, it is possible that you

won't find second order must set (like in this tutorial). You can check if the algorithm is working by defining excludedRxns as an empty array. If the algorithm is working well, you should see how the must sets are found in each iteration.

2) problem: "I got an error when running the findMust functions"

possible reason: inputs are not defined well or GAMS was not installed correctly

solution: verify your inputs. To verify that GAMS was installed correctly, in MATLAB, use the function `which('gams')`. If a folder is shown, GAMS was installed correctly.

ANTICIPATED RESULTS

In this tutorial some folders will be created inside the folder called "runID" to store inputs and outputs of the optForce functions (`findMustUWithGams.m`, `findMustLWithGams.m`, `findMustUUWithGams.m`, `findMustLLWithGams.m`, `findMustULWithGams.m`, `optForceWithGams.m`)

In this case `runID = 'TestOptForce'`, so inside this folder the following folders will be created:

CurrentFolder

```
|   TestOptForce
|   |   InputsFindMustL
|   |   OutputsFindMustL
|   |   InputsFindMustU
|   |   OutputsFindMustU
|   |   InputsFindMustLL
|   |   OutputsFindMustLL
|   |   InputsFindMustUU
|   |   OutputsFindMustUU
|   |   InputsFindMustUL
|   |   OutputsFindMustUL
|   |   InputsOptForce
|   |   OutputsOptForce
```

The input folders contain inputs (.txt and .gdx files) for running the GAMS functions to solve each one of the bilevel problems. Output folders contain results of the algorithms (.xls and .txt files) as well as a report (.txt) summarizing the outcomes of the steps performed during the execution of the optForce functions. Additionally,

some auto-generated files will be stored (.gdx and .lst files). These files are generated by GAMS automatically and contain values for variables and the summary of the GAMS execution, respectively.

The optForce algorithm will find sets of reactions that should increase the production of your target. The first sets found should be the best ones because the production rate will be the highest. The last ones should be the worse because the production rate will be slower. Be aware that some sets could not guarantee a minimum production rate for your target, so you always have to check the minimum production rate. You can do this using the function testOptForceSol.m. Some sets could allow a higher growth rate than others, so keep in mind this too when deciding which set is better.

Acknowledgments

I would like to thank the research group of Costas D. Maranas who provided the GAMS functions to solve this example. In particular I would like to thank to Chiam Yu Ng who kindly provided examples for using GAMS and support for writing GAMS functions.

References

- [1] Ranganathan S, Suthers PF, Maranas CD (2010) OptForce: An Optimization Procedure for Identifying All Genetic Manipulations Leading to Targeted Overproductions. PLOS Computational Biology 6(4): e1000744. <https://doi.org/10.1371/journal.pcbi.1000744>.
- [2] Maciek R. Antoniewicz, David F. Kraynie, Lisa A. Laffend, Joanna González-Lergier, Joanne K. Kelleher, Gregory Stephanopoulos, Metabolic flux analysis in a nonstationary system: Fed-batch fermentation of a high yielding strain of E. coli producing 1,3-propanediol, Metabolic Engineering, Volume 9, Issue 3, May 2007, Pages 277-292, ISSN 1096-7176, <https://doi.org/10.1016/j.ymben.2007.01.003>.