IN SILICO ENGINEERING OF BIOCHEMICAL NETWORK OF *ZYMOMONAS MOBILIS* ADAPTATION FOR GLYCEROL CONVERSION INTO BIOETHANOL

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Abstract. One of the perspectives of rural development is the production of biofuels. One of the biofuel production problems is a significant quantity (about 10 %) of a by-product – glycerol occurrence. This problem is offered to solve by adaptation of bacteria *Zymomonas mobilis*, which is notable for ethanol production facilities. To be able to process glycerin into ethanol using *Z.mobilis* bacteria, the bacteria must be modified, because its natural form cannot process glycerin. At the same time computer modelling analysis is required, to assess specific modification affectivity in interconnection with other processes in bacteria. The computer model, which describes two genes of **-** bacteria E.Coli GlpF and GlpK insertions and expressions in bacteria *Zymamonas mobilis*, describes conversion of glycerol into bioethanol in *Z.mobilis* bacterial cell. Biochemical reactions and the process regulation network are too complicated, to be able to predict the system response without extensive computer modelling by changing any of its components. The first phase of the model creation was creation of a structure model based on biochemical reactions. The second phase of model creation is stoichiometry analysis. The stoichiometry analyzed the possible steady states and reactions flux. Using the databases KEGG, SABIO-RK, BRENDA, ChEBI are defined reactants, reactions. Stoichiometry analysis of biochemical network of *Zymomonas mobilis* adaptation for glycerol conversion into bioethanol was created using program COBRA Toolbox.

Keywords: *Zymomonas Mobilis*, Stoichiometry analysis.

Introduction

Systems biology is a rapidly growing science field that is based on building and validating in silico models of biological systems. Under different environmental conditions and genetic backgrounds the mathematical constraint – based modeling approach of metabolism is used to predict an optimal metabolic yield and steady state flux distributions [1]. A cell metabolism is a highly branched network of intracellular chemical reactions. It contains numerous intracellular compounds such as metabolites, enzymes, nucleotides, cofactors, redox [(NAD(P)(H)], and energy (ATP) carriers which take part in many anabolic and catabolic pathway reactions. The coordinated regulation of this reaction network is implemented through numerous transcriptional, translational, allosteric, and feedback regulatory mechanisms. Optimizing metabolic flux towards the desired end product(s) requires understanding how the non-linear enzymatic rate equations are impacted by heterologous enzyme expression levels. A rigorous kinetic modeling framework must consider non-linearities in the mechanistically derived Michaelis–Menten rate expressions, including the feedback regulations. This is a significant limitation of the current metabolic flux analysis or control theory approaches [2-6] and biochemical systems approaches [7] based on linearized methodologies. [8]

Z mobilis is undoubtedly one of the unique micro-bacteria in the world. It is known since 1912 as *Termobacterium mobilis*, Pseudomonas Linder, and finally as *Z.mobilis*. The first reviewing of their uniqueness was published in 1977 and 1988. *Z.mobilis* features manifest not only in biochemistry but also in growth, energy production, and response to the growing conditions. These features caused great interest in science, biotechnology, and industrial areas. *Z.mobilis* is a bacterium which is notable for ethanol production facilities. One of the biofuel production problems is a significant quantity (about 10 %) of a by-product – glycerol occurrence. This problem is offered to solve by adaptation of bacteria *Z. mobilis*, which is notable for ethanol production facilities. [9]

Biochemical reactions and the process regulation network are too complicated to be able to predict the system response without extensive computer modelling after changing any of its components.

The first step of creating dynamic models is stoichiometry analysis. Stoichiometry is a branch of chemistry that deals with the quantitative relationships that exist between the reactants and products in chemical reactions. In a balanced chemical reaction, the relations among the quantities of reactants and products typically form a ratio of whole numbers. Stoichiometry analysis uses matrix algebra to deduce the constraints implicit in metabolic networks. When applied to simple networks, it can often

give the impression of being an unnecessarily complicated way of arriving at information that is obvious from inspection. [2] Stoichiometry analysis can be made with program COBRA Toolbox.

Materials and methods

Stoichiometry analysis of biochemical network of *Zymomonas mobilis* adaptation for glycerol conversion into bioethanol [10] can be made with program COBRA Toolbox. [11] COBRA Toolbox is software for in silico metabolic network reconstruction creation and usage of different analysis methods. All metabolites and reactions data must satisfy each specific requirement for software analysis methods. With COBRA Toolbox the flux balance analysis and robustness analysis can be done. The flux balance analysis is based on the optimization of an objective function, which is used as an evaluation criterion to identify an optimal flux distribution among all possible steady state flux distributions that meet the objective. The robustness analysis is performed by varying a particular flux over a specified range of values and recalculating the objective function. As the resulting curve depicts the sensitivity of the objective function to that particular flux, the robustness analysis can be used to assess the effect of reducing flux trough particular reaction on the given objective [12].

Metabolic network was performed by stoichiometrics analysis containing 20 reactions, 6 exchange reactions, 28 metabolites. COBRA Toolbox uses .XLS format file to store additional information into each metabolite and each reaction sheet [5; 13]. All this information for metabolites and reactions must be taken from databases and tools on the Internet [14] and completed in .XLS file without disturbing the file structure. This completed file is loaded with COBRA Toolbox commands in Mathlab, where this file is tested for structural or syntactical errors. One of the main conditions is that all reactions must be balanced and all metabolites must have a correctly assigned chemical formula. When the above motioned steps have been reached, the metabolic network data have been correctly conformed, only then we can make the metabolic network model analysis.

Reactants, reactions, reactants neutral and charge formula were taken from publications M.M.Altintas et al. model [8], B.M.Bekker et al. [11], W.J.Colin et al. [15] and this information was proofing in the database KEGG (http://www.genome.jp/kegg/), BRENDA (http://www.brenda-enzymes.org/), ChEBI (http://www.ebi.ac.uk/chebi/aboutChebiForward.do), SABIO-RK (http://sabio.villa-bosch.de/#Intro), BIGG (http://bigg.ucsd.edu/) [13].

Results and discussion

Analyzing the stoichiometric of the model, we are interested in possible steady state, reactions flux, limited reactions fluxes, if the objective function is determined. The determined function of this model is ethanol flux (‘Transport_etoh[e]’), this reaction maximal flux is achieved +1000 unit – $mmol \cdot gDW^{-1} \cdot hr^{-1}$ (millimoles per gram dry cell weight per hour, the default flux units used in the COBRA Toolbox). The reaction flux interval lower bound and upper bound were defined. Irreversible reactions have lower bound 0 unit – $mmol \cdot gDW^{-1} \cdot h^{-1}$ and upper bound +1000 unit – $mmol \cdot gDW^{-1} \cdot h^{-1}$, and reversible reactions have lower bound -1000 unit – $mmol \cdot gDW^{-1} \cdot h^{-1}$ and upper bound +1000 unit – $mmol \cdot gDW^{-1}\ h^{-1}$.

Using linear algebra methods are calculated reactions fluxes, at witch will be achieved the objective function and model has steady state. Through the flux balance analysis steady state with the following reaction fluxes is derived (Table.1). Table 1 contains information about the results of simulations. The column *Growing on Glucose* – bacterial cell is growing only to substrate glucose (‘Transport_glc-D[e]’), flow in the glycerin (‘Transport_glyc[e]’) branch (‘Transport_glyc[e]’, ‘GLYK’, ‘G3PD1’, ‘TPI’, ‘Glyc_tr’, ‘Oxyg’) is 0 unit – $mmol \cdot gDW^{-1} \cdot h^{-1}$. Towards the objective function (‘Transport_etoh[e]’) glucose reaction flux must be at least +500 unit – $mmol \cdot gDW^{-1} \cdot h^{-1}$, the result, from one glucose unit two units of ethanol can be obtained.

The column *Growing on Glycerol* – bacterial cell is growing only to substrate glycerin (‘Transport_glyc[e]’), flow in the glucose (‘Transport_glc-D[e]’) branch (‘Transport_glc-D[e]’, ‘GK’, ‘GPD’, ‘PGLS’, ‘PGD’, ‘KDPGA’) is 0 unit – $mmol \cdot gDW^{-1} \cdot h^{-1}$. The result, grow only on glycerol-based, is not achieved the maximum results of the obtained function ‘Transport_etoh[e]’=+1000 unit – $mmol \cdot gDW^{-1} \cdot h^{-1}$. In this case, potential factors can be found, which are affecting the results. Through simulations with this model, it was possible to clarify reactions, which affected the results. The

reactions 'ATP' reached maximum of flux, and if the reaction flux interval increases, then the model can achieve the desired result.

The column *Combining Glucose & Glycerol* – bacterial cell is growing to substrate glucose and substrate glycerin. The objective function is achieved with glucose flux ('Transport_glc-D[e]') +250 unit – mmol·gDW^{-1}·h^{-1} and glycerin flux ('Transport_glyc[e]') +500 unit – mmol·gDW^{-1}·h^{-1}.

Table 1

Results of flux balance analysis

Reaction	Growing on Glucose	Growing on Glycerol	Combining Glucose & Glycerol
'GK'	500	0	250
'GPD'	500	0	250
'PGLS'	500	0	250
'PGD'	500	0	250
'KDPGA'	500	0	250
'GAPD'	500	666	750
'G3PK'	500	666	750
'GMP'	-500	-666	-750
'ENO'	500	666	750
'PYRK'	500	666	750
'PYRD'	1000	666	1000
'ADH'	1000	666	1000
'GF'	500	0,00	250
'ETOHext'	1000	666	1000
'ATP'	500	**1000**	1000
'GLYK'	0	666	500
'G3PD1'	0	666	500
'TPI'	0	666	500
'Glyc_tr'	0	666	500
'Oxyg'	0	333	250
'Transport_glc-D[e]'	**500**	**0**	**250**
'Transport_etoh[e]'	**1000**	**666**	**1000**
'Transport_co2'	1000	666	1000
'Transport_glyc[e]'	**0**	**666**	**500**
'Transport_o2'	0	333	250
'Transport_h2o'	0	666	500

Through the alternative flux balance analysis the interval of the reaction flux when the model has steady state was revealed. If the determined function of this model is ethanol flux ('Transport_etoh[e]'), these reactions maximal flux is achieved +1000 unit – mmol·gDW^{-1}·h^{-1}, the results are shown in Figure 1.

The robustness analysis results debt (Fig. 2.): a) if bacterial cell is growing only with substrate glucose, then maximal ethanol flux ('Transport_etoh[e]'= +1000 unit – mmol·gDW^{-1}·h^{-1}) is achieved already by glucose flux ('Transport_glc-D[e]') constrain +250 unit – mmol·gDW^{-1}·h^{-1}, when the glucose flux increases, then it will be also steady state with maximal flux; b) if bacterial cell is growing with substrate glycerin, then maximal ethanol flux is achieved with glycerin flux ('Transport_glyc[e]') constrain is +500 unit – mmol·gDW^{-1}·h^{-1}, increasing glycerin flux then in the cell inhibitions begin; c) if bacterial cell is growing with substrate glucose (if glucose flux constrain is constant +250 unit – mmol·gDW^{-1}·h^{-1}) and substrate glycerin, then maximal ethanol flux is achieved with glycerin flux ('Transport_glyc[e]') constrain is +500 unit – mmol·gDW^{-1}·h^{-1}, increasing glycerin flux then in the cell inhibitions begin.

```
ans =

    'Reaction Name'          'Min flux'       'Max flux'    'Detailed view'
    'GK'                     [      250]     [      500]         {2x2 cell}
    'GPD'                    [      250]     [      500]         {2x2 cell}
    'PGLS'                   [      250]     [      500]         {2x2 cell}
    'PGD'                    [      250]     [      500]         {2x2 cell}
    'KDPGA'                  [      250]     [      500]         {2x2 cell}
    'GAPD'                   [      500]     [      750]         {2x2 cell}
    'G3PK'                   [      500]     [      750]         {2x2 cell}
    'GMP'                    [     -750]     [     -500]         {2x2 cell}
    'ENO'                    [      500]     [      750]         {2x2 cell}
    'PYRK'                   [      500]     [      750]         {2x2 cell}
    'PYRD'                   [     1000]     [     1000]         {2x2 cell}
    'ADH'                    [     1000]     [     1000]         {2x2 cell}
    'GF'                     [      250]     [      500]         {2x2 cell}
    'ETOHext'                [     1000]     [     1000]         {2x2 cell}
    'ATP'                    [      500]     [     1000]         {2x2 cell}
    'GLYK'                   [        0]     [      500]         {2x2 cell}
    'G3PD1'                  [1.7053e-013]     [500.0000]         {2x2 cell}
    'TPI'                    [1.7053e-013]     [500.0000]         {2x2 cell}
    'Glyc_tr'                [        0]     [      500]         {2x2 cell}
    'Oxyg'                   [        0]     [      250]         {2x2 cell}
    'Transport_glc-D[e]'     [      250]     [      500]         {2x2 cell}
    'Transport_etoh[e]'      [     1000]     [     1000]         {2x2 cell}
    'Transport_co2'          [     1000]     [     1000]         {2x2 cell}
    'Transport_glyc[e]'      [        0]     [      500]         {2x2 cell}
    'Transport_o2'           [        0]     [      250]         {2x2 cell}
    'Transport_h2o'          [        0]     [500.0000]         {2x2 cell}
```

Fig. 1. **Results of flux balance analysis – alternative fluxes**

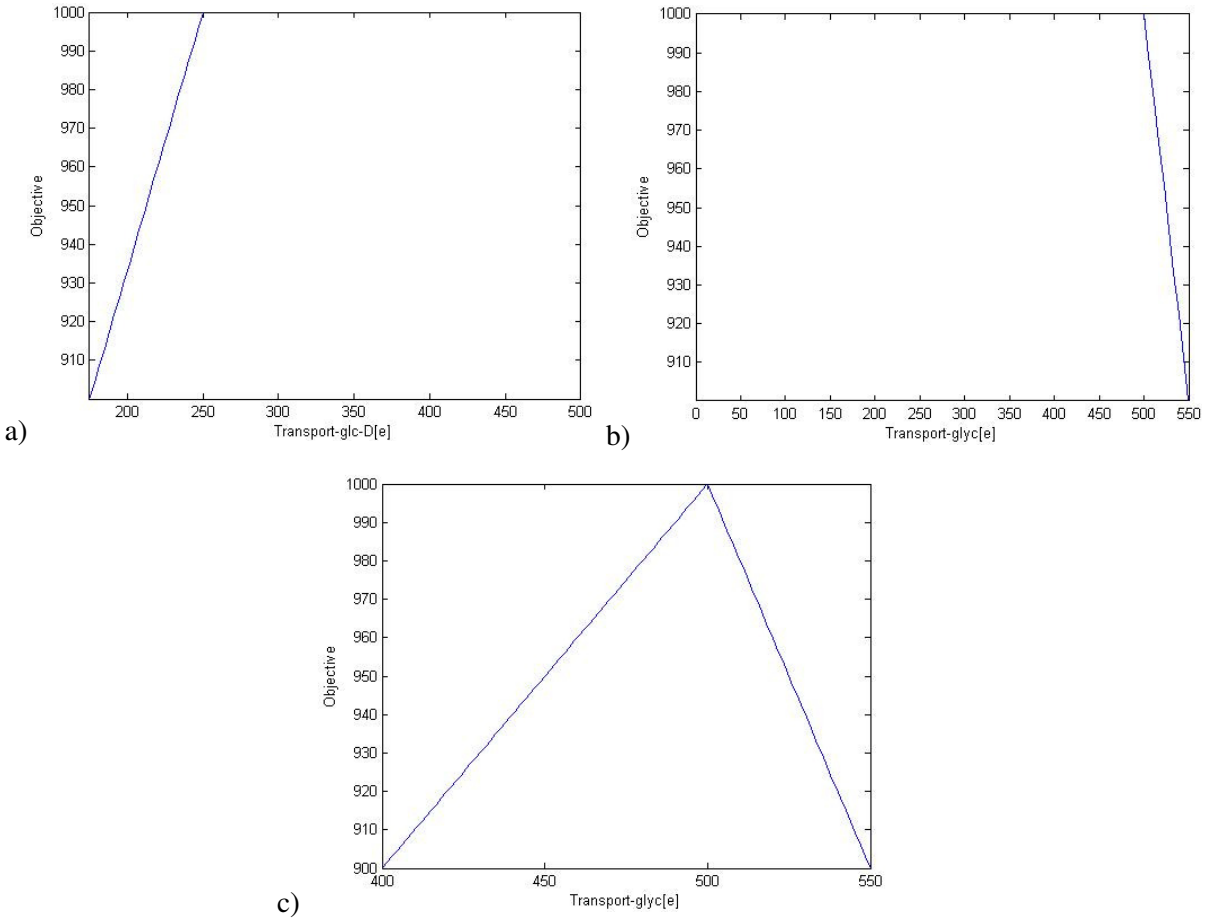

Fig. 2. **Robustness analysis results**

Conclusions

Cobra Toolbox for steady state metabolic network models analysis is provided for small and greater metabolic networks with hundreds or thousands reactions. It allows changing unlimited count manipulating and optimizing reactions fluxes. COBRA Toolbox is a great calculation tool for enormous amount of data.

The computer model which describes conversion of glycerol into bioethanol in Z.mobilis bacterial cell has several steady states. Consequently, the next step can create a dynamic model, having regard to the reaction flux relations, which were identified by stoichiometrics analysis.

Stoichiometry analysis showed that if the bacterial cell is growing only to substrate glycerin, then to achieve maximum ethanol production, ATP productions in the bacterial cell increased. The results show that the result will be better if the cell will be growing with substrates glucose and glycerin.

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