original scientific article

Assessment of *Zymomonas mobilis* biotechnological potential in ethanol production by flux variability analysis

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Abstract: *Metabolic engineering of microorganisms is a promising direction in industrial biotechnology. Engineering approach requests finding the best possible alternative of metabolic pathway fluxes, with preset limitations for productivity, yield, set of by-products, robustness and other features. In case of industrial applications the steady state precondition has to be taken into account, thus enabling application of stoichiometric modeling. Spectrum of solutions maximizing ethanol or succinate production from glucose or xylose is analyzed at fixed maximal production rate of products and for 80-100% range of maximal production rate to assess the robustness of yield to disturbances in the performance of reactions. Maximal production of desired components from glucose and xylose turned out to be very similar by the flux distribution with very limited ability to compensate throughput limitations of important reactions thus causing reduction of yield. Requesting the network performance at 80-100% of yield leads to increase of robustness, and majority of reactions can be compensated by others to maintain the steady state at the desired yield level.*

1. Introduction

Ethanol and succinate production (Blank and Kuepfer, 2010; Dien et al., 2003; Hübner et al., 2011; Lee et al., 2010; Patil et al., 2004) by metabolically engineered microorganisms (Nielsen and Keasling, 2011; Pharkya and Maranas, 2006) is a perspective direction in biotechnology.

Often when optimizing flux distribution, only the best flux is assessed (Pentjuss, et al., 2013a). More advanced approach is to look for the best possible flux distribution, when biomass growth is taking place, to make the bioconversion more efficient. Another step forward is to ensure that even the worst possible flux distribution at growing biomass conditions give industrially feasible yield (Trinh et al., 2008). That can be done using elementary mode analysis (Trinh et al., 2009) or flux variability analysis (FVA) (Boele et al., 2012; Orth et al., 2010). Elementary mode analysis requests cumbersome calculations, while FVA can be performed with small computational effort (Boele et al., 2012; Gudmundsson and Thiele, 2010; Kostromins, 2012; Orth et al., 2010; Pentjuss, et al., 2013b), and gives good overview of the range of possibilities and stability of possible solutions. Particular solutions can be visualized at different scales (Kostromins and Stalidzans, 2012) and analysed more closely.

Recent progress with stoichiometric modeling of bacteria *Zymomonas mobilis* enables application of the FVA approach. The first stoichiometric models of *Z. mobilis* were published without available metabolic model files (Lee et al., 2010; Tsantili et al., 2007; Widiastuti et al., 2011) and therefore were not directly applicable for modeling and optimization. In this study the stoichiometric model of *Z. mobilis* central metabolism (Pentjuss, et al., 2013a) is used. The model does not include the fluxes from the central metabolism to biomass growth, and therefore would be valid for non-growing cells, e.g. for immobilized culture. So far for this model published optimization results (Pentjuss, et al., 2013a) show just one of the optimal solutions without analysis of the whole spectrum of possible flux distributions.

Still it has to be noted that stoichiometric modeling does not take into account thermodynamic features of enzymes and realistic production rate depends on the values of kinetic parameters (Kalnenieks et al., 2014). Therefore, the outcomes of stoichiometric modeling have to be treated as the yield values that definitely can not be exceeded, but could remain unreachable.

In this study we aim to apply a more detailed approach to the central metabolism model of *Z. mobilis* (Pentjuss, et al., 2013a) to discover the full spectrum of optimal solutions and their robustness in terms of alternatives in flux distributions for production of ethanol and succinate from glucose and xylose.

2. Materials and methods

2.1. Model of *Z.mobilis* central metabolism and task setting

Stoichiometric model of *Z. mobilis* central metabolism (Pentjuss, et al., 2013a) as published in supplementary materials (Pentjuss, et al., 2013a) is used for optimization of ethanol and succinate production from glucose and xylose. The model includes 58 reactions of central metabolic pathways and 21 transmembrane transport reactions. Additionally, 21 virtual

exchange reactions are introduced according to the protocol of model development (Thiele and Palsson, 2010). For analysis purpose 4 reactions, present in the published reconstruction of the central metabolism (Pentjuss, et al., 2013a) were knocked out (Glycerate kinase (E.C. 2.7.1.31), heterologous 3-phosphoglycerate phosphatase (E.C. 3.1.3.38), citrate lyase (E.C. 4.1.3.6) and 6-phosphogluconate dehydrogenase (E.C. 1.1.1.44)). Also, model was extended with additional reactions (glycerol-3-phosphate dehydrogenase [NADH+] E.C. 1.1.1.8 (G3PD2), glutamate dehydrogenase E.C. 1.4.1.3 (GD2), malate dehydrogenase (oxaloacetate-decarboxylating) NADP+ E.C. 1.1.1.40 (MEMDLD)) to cover the possible redox-balancing reactions in *Z. mobilis*.

FVA was performed at substrate input flux of 100 µ mol gDW^{-1} hr^{-1} at: 1) maximal value of product yields and 2) 80-100% of maximal value of desired product yields. Four substrate-product pairs were evaluated: glucose-ethanol, glucose-succinate, xylose-ethanol and xylose-succinate.

2.2. Analysis methods and software tools

ScrumPy software (Poolman, 2006) was used for modeling and analysis. Flux variability analysis (Burgard et al., 2001) method was implemented into ScrumPy. Visualization was carried out by Linux compatible graphic utility Gnuplot (http://www.gnuplot.info/) with Gnuplot.py (http://gnuplot-py.sourceforge.net/) library using CandleSticks option.

Alternatively, the experiment could be performed also by COBRA Toolbox 2.0 (Schellenberger et al., 2011).

3. Results and discussion

The classical flux variability analysis goal is to get an overview about the possible flux distributions at the maximal yield of the desired product (Fig. 1 and Fig. 2). The 54 central metabolic pathways reactions are placed on the left side of the horizontal axis of figures followed by 19 transmembrane transport reactions. The virtual exchange reactions are not introduced in the figures. The variability of reactions is summarized in Table 1.

3.1. Flux variability at maximal production rate of products

Maximal yield of products can be determined by optimizing production of a particular product and setting that value as a requested limitation during flux variability analysis.

3.1.1. Production of ethanol

Flux variability analysis at maximal production yield of ethanol demonstrates just seven reactions for substrates with rates that may vary in a relatively wide range. Thus, reduction

of flux of one of those reactions may be compensated by one or more of the others. The difference between glucose and xylose as substrates is small as it can be seen in Fig. 1a and Fig. 1b.

There are 31 and 30 reactions without flux for glucose and xylose correspondingly. At the same time the number of variable reactions within 10 µmol gDW^{-1} hr^{-1} are 10 and 3 for glucose and xylose, correspondingly. That indicates a higher robustness of ethanol production from glucose, than from xylose.

3.1.2. Production of succinate

Flux variability analysis at maximal production yield of succinate (Fig. 1c and Fig. 1d) indicates significant variability opportunities of 13 reactions for both substrates. There are 29 and 8 reactions without flux for glucose and xylose correspondingly indicating higher metabolic load and lower robustness in case of xylose. At the same time the number of variable reactions within 10 µmol gDW^{-1} hr^{-1} are 2 and 14 for glucose and xylose, correspondingly, indicating higher robustness in case of xylose.

3.2. Flux variability at 80-100% of maximal yield of products

To see the robustness of the system under lower yield of products, the FVA was repeated, setting the upper/lower boundary limits of products to 80-100% of maximal yield. Regarding the byproducts spectrum, there are very wide options: acetate, acetoin, acetaldehyde, pyruvate, glutamate, succinate, dihydroacetone.

3.2.1. Production of ethanol

During production of ethanol at 80-100% of maximal yield (Fig. 2a and Fig. 2b) there are 35 reactions with significant variability for both substrates. Just 4 and 11 reactions have a fixed non-zero value for glucose and xylose as substrates, respectively, indicating the small level of robustness. There are 15 and 8 reactions with zero value for glucose and xylose, correspondingly, showing the number of reactions with no influence on product flux.

3.2.2. Production of succinate

The production of succinate at 80-100% level of maximal yield (Fig. 2c and Fig. 2d) also becomes much more robust, than at set maximal product yield, having acetate, acetoin, acetaldehyde, pyruvate, glutamate, succinate, dihydroacetone as the possible byproducts. The summary of variability of succinate production (Table 1) is identical to the one for ethanol for both substrates.

Table 1.

Variability of 54 central metabolic network reactions depending on substrate-product pair. Transmembrane transport reactions are not included.

| Substrate-product pair	Yield (% of max)	Reactions with variability above 10 µmol gDW^{-1} hr^{-1}	Reactions with variability below 10 µmol gDW^{-1} hr^{-1}	Constant nonzero flux	No flux	Total reactions except substrate and product
Glucose-ethanol	100	7	10	6	31	54
Xylose-ethanol	100	7	3	14	30	54
Glucose-succinate	100	13	2	10	29	54
Xylose-succinate	100	13	14	19	8	54
Glucose-ethanol	80-100	35	0	4	15	54
Xylose-ethanol	80-100	35	0	11	8	54
Glucose-succinate	80-100	35	0	4	15	54
Xylose-succinate	80-100	35	0	11	8	54
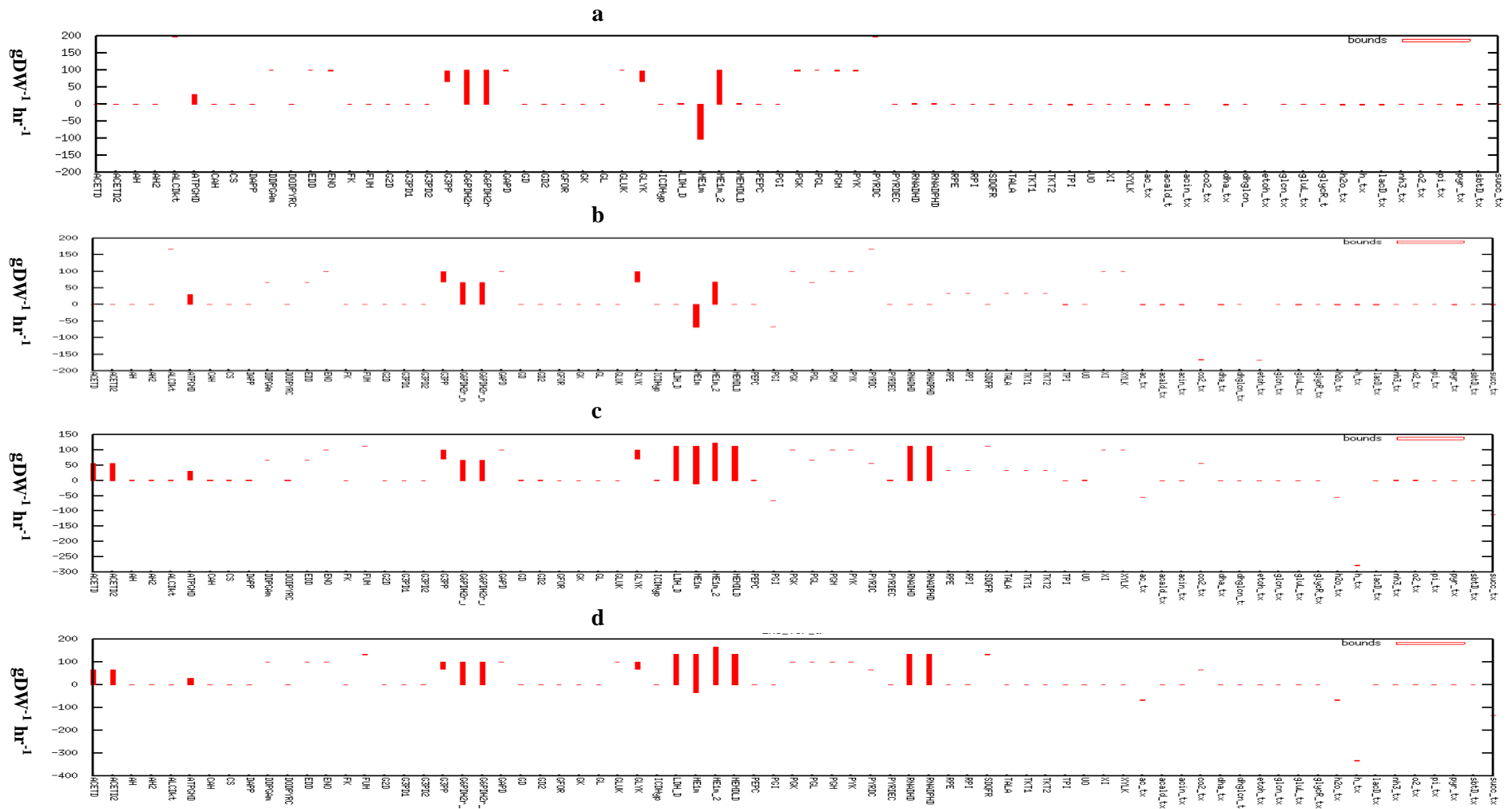
Fig. 1. **Flux variability analysis for substrate-product pairs glucose-ethanol (a), xylose-ethanol (b), glucose-succinate (c), xylose-succinate (d) at maximal product production and fixed substrate consumption rate 100 μmol gDW^{-1} hr^{-1}.**
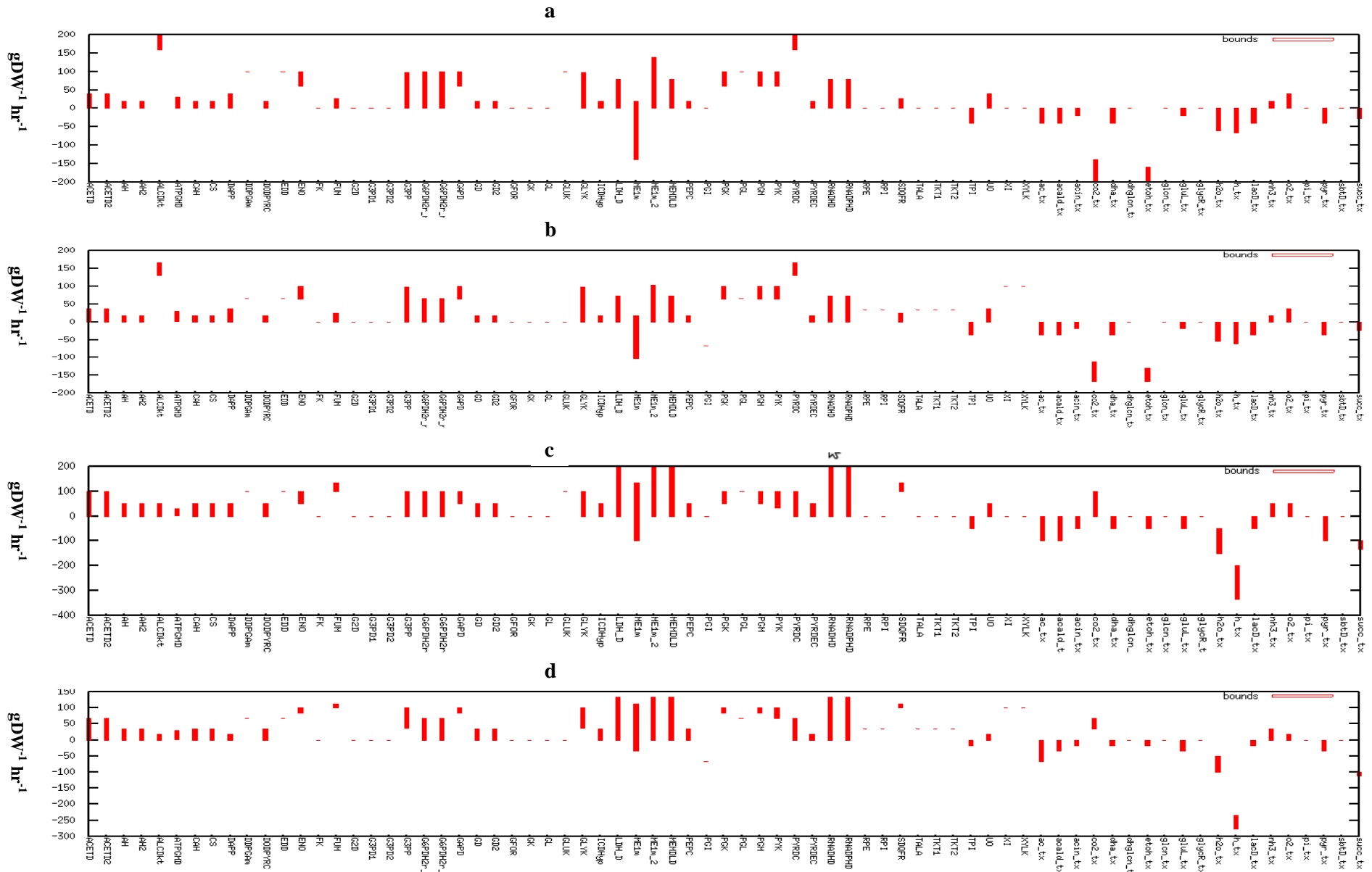
Fig.2. **Flux variability analysis for substrate-product pairs glucose-ethanol (a), xylose-ethanol (b), glucose-succinate (c), xylose-succinate (d) at 80-100% of maximal product production and fixed substrate consumption rate 100 μmol gDW^{-1} hr^{-1}**

3.2.3. Comparative robustness of substrate-product pairs

As expected the robustness or tolerance to failures or flux reductions of different reactions increase if the requested product yield range is widened. Among the 100% yield substrate-product pairs the most robust seems to be the combination is xylose-succinate pair with 27 variable reactions. At the same time the low number of no flux reactions decreases the robustness of yield.

Widening the requested yield range to 80-100% from maximal the number of variable reactions increases up to 35 for all four combinations and there are no reactions with variability below 10 µmol gDW^{-1} hr^{-1}. Glucose as a substrate give more robustness having 15 zero flux reactions in contrast to xylose-based processes with 8 zero flux reactions.

4. Conclusion

Concentration on analysis of maximal yield of the desired product excludes the issue of robustness from analysis, although it is very important in industrial environment. Flux variability analysis (FVA) is one of the tools to assess the robustness and fragility of the metabolic process of interest. Main indicators for robustness are the number of reactions varying in wide range and the number of zero flux reactions, while the number of constant nonzero flux and reactions with low variability indicate fragility of the process of interest.

At maximal yield of products there often are very limited alternatives for the main production pathway. Reaching maximal product yield both in case of ethanol and succinate production *Z. mobilis* metabolic network shows small redundancy in network flux distribution, but lowering the requested ethanol or succinate production yield, the redundancy in byproduct fluxes increases. The number of reactions with variability above 10 µmol gDW^{-1} hr^{-1} increases from 7 and 13 for ethanol and succinate production to 35 widening the requested range of yield from 100% to 80-100%.

Reduction of the requested product yield and varying the set of byproducts in the model might be a reasonable approach to keep the economical benefits at acceptable level and increase the redundancy of the microbial producer strain.

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