



Metabolic Modelling of Itaconic Acid Fermentation with *Ustilago Maydis*

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Itaconic acid (IA) is considered as an important intermediate in the synthesis of bio-based chemicals and fuels. It can be produced under nitrogen-limited conditions from glucose by aerobic fermentation with the fungus *Ustilago maydis* (*U. maydis*). A metabolic model of this organism is developed to identify optimal operating conditions for a continuous fermentation which maximizes the overall itaconic acid yield. Experimental studies show that IA is only produced under nitrogen limitation. In order to capture this behaviour, the model should not only include the common metabolic pathways such as glycolysis, the pentose phosphate pathway and the tricarboxylic acid cycle, but it should also comprise the amino acid metabolism as well as nucleotide and chitin synthesis. Since it has been shown experimentally that nitrogen limitation leads to changes in the elemental composition of the biomass, this effect must also be considered in the model. In this contribution, we will present a metabolic model accounting for these phenomena and compare model predictions with experimental results. A metabolic flux analysis carried out with the model clearly shows that nitrogen-free compounds such as IA acid are expressed by an overflow metabolism as soon as a nitrogen limitation occurs.

1. Introduction

Sustainable routes for the production of novel biofuels are explored in the cluster of excellence “Tailor-Made Fuels from Biomass (TMFB)” at RWTH Aachen University (Wimmer, 2007). In contrast to many other biofuel projects, the research strategy in the TMFB cluster aims at preserving the native structure of biomass to the extent possible during the molecular transformation of the raw materials to fuel molecules. Candidate fuel molecules are carefully chosen to achieve high efficiency and low pollutant emission in novel low-temperature combustion engines (Janssen et al., 2010; Hechinger et al., 2010). Recently, 3-methyltetrahydrofuran (3-MTHF) has been confirmed to be a promising fuel component (Voll and Marquardt, 2011). Itaconic acid (IA) constitutes an important intermediate in the synthesis route of 3-MTHF (Geilen et al., 2010). Besides, this dicarboxylic acid has also been listed as one of the future building blocks for the production of bulk and specialty chemicals from biomass by Werpy and Petersen (2004) and Lee et al. (2011). Currently, IA is produced by fungal fermentation with *Aspergillus terreus* (*A. terreus*) (Okabe et al., 2009), but the productivity and yield must be significantly improved to reduce the production cost to meet the economical constraints of a low priced fuel precursor. Research in the TMFB cluster concentrates at a continuous process, where IA is converted from glucose by aerobic fermentation with the corn smut fungus *Ustilago maydis* (*U. maydis*). This fermentation has to

run under optimal operating conditions to maximize the IA yield. To this end, experimental investigations are accompanied by modelling activities. In particular, a metabolic model of *U. maydis* is developed to better understand the IA production pathways and to obtain useful hints for potential genetic engineering. Moreover, the metabolic model can also be integrated into a fermenter model such that the processing conditions can be fully linked to the metabolic level. Such a comprehensive model not only facilitates process analysis and optimization by a targeted design of fermentation experiments, but it will also support process and control system design in the future.

2. Itaconic acid fermentation

Currently, most IA is produced on industrial scale by fermentation with the fungus *A. terreus*. Worldwide, more than 80,000 tons of IA are produced per annum (Okabe et al., 2009). In general, low cost raw materials including molasses and hydrolysed starch are fermented in batch cultivations under phosphate limitation and at low pH values. Although high final concentrations of IA up to 80 g/L were reported for *A. terreus* (Yahiro et al., 1995), the filamentous growth of this fungus imposes restrictions on the process. In addition to the sensitivity of the filamental pellets to hydro-mechanical stress, they are known to inhibit mass transfer and consequently oxygen supply of the cells.

The research goal in the TMFB cluster is to convert cellulosic biomass directly to IA in a continuous simultaneous saccharification and fermentation (SSF) process using *U. maydis*. This fungus grows like yeast in single cells and produces IA at moderate pH values (Haskins et al., 1955). Hence, the hydrolysis of cellulose with common cellulases and the fermentation from glucose to IA are possible in a single bioreactor. We first characterized a non-optimized *U. maydis* wild type strain in small-scale cultivations to investigate its growth and production capacity using glucose as a substrate. These experiments have confirmed the expectation that *U. maydis* is a robust microorganism which is capable to produce IA from hydrolysed lignocellulosic raw materials. In these small-scale experiments, nitrogen limitation was shown to be a crucial prerequisite for IA production with *U. maydis* (Klement et al., 2012). Hence, the batch fermentation can be clearly divided into a trophophase of unlimited microbial growth and an idiophase with nitrogen-limited production of IA. After nitrogen had been depleted in the cultivation medium, the elemental composition of the fungal biomass changed in subsequent hours. In particular, the carbon-to-nitrogen-ratio of the biomass was shown to increase significantly, which could be attributed to the production of large amounts of storage (glyco-)lipids. As high concentrations of IA above 25 g/L were proven to inhibit growth as well as production, a continuous fermentation with *U. maydis* in a stirred tank bioreactor of 2 L was developed. These continuous fermentations were performed in a defined mineral medium at a temperature of 30 °C and a constant pH value of 6.0. The results of these experiments will be compared to model predictions in the “Results” section below.

In continuous fermentation, a given dilution rate is applied by feeding fresh medium to and removing the same volumetric amount of culture broth from the bioreactor. In contrast to batch cultivations, the biomass leaving the reactor in the effluent has to be balanced by a continuous growth of the fungus at steady state. As nitrogen is essential for biomass production, low amounts of nitrogen have to be fed to the culture. Steady-state conditions are established after the start-up transient: then, all concentrations in the bioreactor, including biomass, product and substrate, are constant over time. These steady-state concentrations of glucose, IA, ammonium and biomass were determined during our experiments of different dilution rates under nitrogen limitation.

3. Metabolic modelling

The experimental results of the continuous fermentations described in the previous section provide the basis for model development. This stationary model should describe growth and production at the same time. The focus of this contribution is on a metabolic model rather than on a complete fermenter model. A comparison of the model-based metabolic flux analysis with the results of the fermentation experiments is possible, if a perfect behaviour of the fermenter is assumed. In particular, it is assumed that no transport limitations exist in the exchange of metabolites between cell and fermentation broth.

The concept of metabolic flux analysis summarizes some well-established techniques to explore the biochemical processes in a particular organism (Varma and Palsson, 1994; Klamt and Stelling, 2003)

by means of mass balances which describe the network of metabolic reactions. Such analyses support, for example, the design of experiments to reveal the structure of a candidate metabolic model, the identification of the differences between diverse operating regimes of the organism or the assessment of the potential for metabolic reengineering. Metabolic networks are formulated in terms of nodes (metabolites) and arcs (reactions) such that stationary mass balances can be set up for each metabolite to describe the material flows through the network. The corresponding model equation can be written as

$$S \cdot v = 0 \quad (1)$$

Here, the matrix **S** holds the stoichiometric coefficients of the metabolic reactions and the vector **v** represents the metabolic fluxes given in mmol of reaction product per gram of dry biomass per hour [mmol/(g·h)]. As this approach normally leads to an underdetermined system of linear equations, optimization strategies can be favourably used to detect an optimal flux distribution or even all alternative optimal pathways (Schilling and Palsson, 1998). Hence, an appropriate objective function must be chosen. Often, the product yield is selected to be maximized.

As detailed pathway information is not available for *U. maydis*, assumptions are either extracted from qualitative investigations - e.g., those of Saavedra et al. (2008) for glycolysis, or are deduced from gene sequence comparisons between *U. maydis* and other fungi (e.g., *Aspergillus niger* (*A. niger*)). The metabolic model of *U. maydis* covers the pathways of the central carbon metabolism for glucose degradation including glycolysis, the glyoxylate and pentose phosphate pathways as well as the tricarboxylic acid cycle. In *A. terreus*, IA is produced from citrate via cis-aconitate by the enzyme cis-aconitate decarboxylase (CAD) (Bonnarme et al., 1995; Tevž et al., 2010). As no specific pathway for IA synthesis in *U. maydis* is available, the pathway of *A. terreus* is postulated to also hold for *U. maydis* and is consequently implemented in the model. Since the fermentation is run under aerobic conditions, the respiratory chain is taken into account. Furthermore, the pathways of the cellular nitrogen metabolism are considered because of experimental evidence that IA is only expressed under nitrogen limitation. Consequently, the model includes the amino acid metabolism as well as the nucleotide and chitin syntheses, since these components represent essential precursors for biomass formation. While the amino acid synthesis is based on the work of McCann and Snetselaar (2008), the reactions for the nucleotide, chitin and biomass formation are taken over from the metabolic pathway of *A. niger* (David et al., 2003). A comparison of model predictions and experimental results indicate that the elemental compositions of the biomass composition do not match. Even though the exact correlation has not yet been identified, the experiments clearly indicate changes in biomass composition with nitrogen concentration and dilution rate. Pragmatically, a linear correlation between biomass composition and these influencing factors has been assumed as a first attempt, with the coefficients being adjusted to match the measured data.

In addition, energy constraints are implemented to represent ATP consumption of the maintenance metabolism and the biomass formation including the synthesis of the macromolecules. Unfortunately, the ATP demands published by different authors differ strongly. Preliminary, the values are fixed to those reported by David et al. (2008), i.e., to 1.9 mol of ATP per g biomass per hour for maintenance and to 46.3 mol of ATP per g biomass for biomass formation. These assumptions have to be checked in the future. In general, nitrogen limitation is assumed to take place, if no accessible ammonium can be detected in the fermentation broth because all ammonium fed is directly consumed by the microorganism. As this definition cannot be directly accounted for in the metabolic model, the nitrogen limitation is described by the ratio of glucose to ammonium uptake. According to the experiments, a nitrogen limitation can be observed at a ratio of approximately 2 (mmol glucose)/(mmol ammonium) and above.

The flux model is complemented by the choice of an appropriate objective function which enormously influences the results of the flux analyses. While a maximization of either IA or biomass could be used to estimate the productivity potential of the IA fermentation, a (weighted) combination of the two will probably best describe the actual behavior in a continuous fermentation. In the following, this latter option is chosen. The ratio of biomass to IA production is specified to a value of 15. This value has shown to lead to the most accurate predictions of the experimental biomass and IA concentrations, if

the glucose and ammonium uptake rates are given. The final model includes 159 reactions and 116 substances, whereas 27 of the products can exist outside of the cells (e.g. IA, CO₂, amino acids, lipids, biomass). The model is implemented and solved in the General Algebraic Modeling System (GAMS).

4. Results

The optimization results obtained from the metabolic model are presented in Figure 1 and compared to the corresponding experimental data. It can be seen that the general trends of the growth rate, the oxygen uptake as well as the IA and CO₂ formation can be predicted reasonably well, if the glucose and ammonium uptake are set to the values compiled in Table 1.

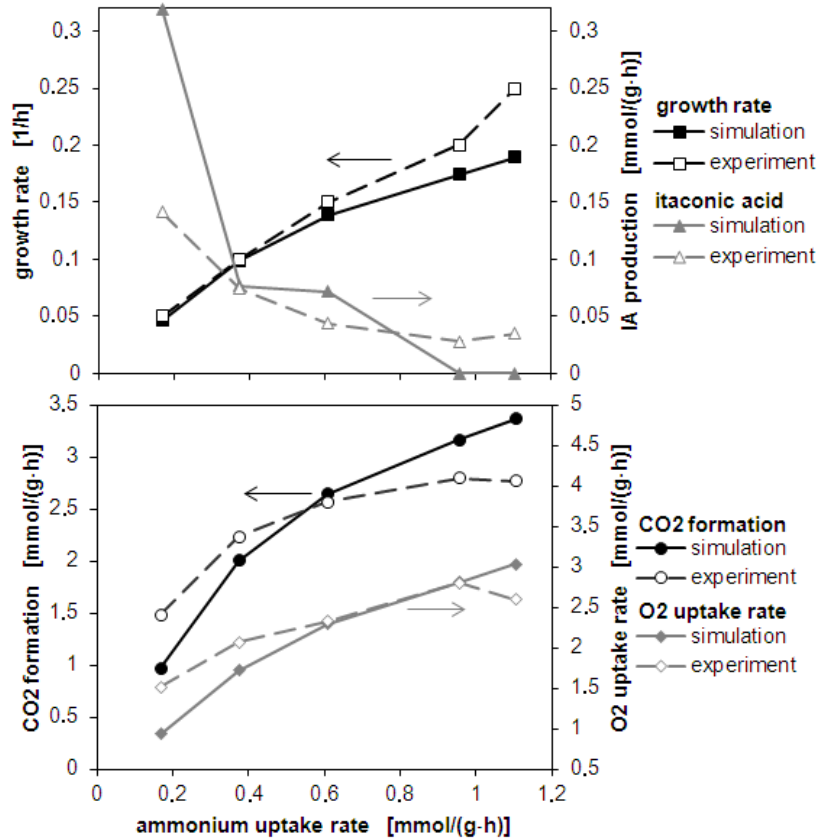


Figure 1: Comparison of simulation and experimental results for IA fermentation with *U. maydis* under nitrogen-limited conditions (lines connecting data points are only shown to guide the eye)

Table 1: Experimental glucose and ammonium uptake rates for the fermentation with *U. maydis*

Ammonium uptake [mmol/(g·h)]	Glucose uptake [mmol/(g·h)]	Glucose-ammonium ratio [-]
0.1686	0.8438	5.0047
0.3749	1.0675	2.9008
0.6087	1.4165	2.3270
0.9573	1.6339	1.7068
1.1058	1.7646	1.5958

In particular, a decreasing IA formation can be observed for an increasing ammonium uptake rate. No IA production is predicted, if the glucose to ammonium uptake ratio falls below 2. Instead, the simulation predicts the formation of nitrogen-containing compounds (e.g. glycine), which could not be validated yet due to a lack of experimental data. It is conjectured, that IA is produced as a consequence of a so-called overflow metabolism, which is known to exist in filamentous fungi (Vrabl et al., 2009). An overflow metabolism occurs, if not all the energy in the cell can be used for biomass synthesis because of a limitation of a non-carbon substrate, while a surplus of the carbon source is supplied. Consequently, other metabolic products are formed to reduce the energy level. In the present case, IA is formed under nitrogen limitation, if glucose is available in excess. The optimization model favours IA production because high IA yield increases the objective function, though other nitrogen-free products (e.g. lipids) could also be generated.

The evaluation of model validity is limited because of experimental shortcomings. Existing experimental capabilities restrict the determination of the product distribution to concentration measurements in the fermentation broth. More insight could be obtained by isotope labelling experiments with ^{13}C -labelled substrates to identify in vivo enzyme activities and metabolic fluxes. A sensitivity analysis of the optimization models clearly indicates that the quantitative predictions strongly depend on the data used in the energy balance, i.e., in the amount of ATP required for maintenance and biomass synthesis. Despite this uncertainty, the influence of the nitrogen limitation and its effects on biomass composition can be captured by this first model at least qualitatively. So far, we refrained from model fitting, because the various model assumptions and consequently the structure of the model have to be carefully checked by additional measurements.

5. Conclusions and future work

In this contribution, we presented a metabolic model for continuous IA fermentation with *U. maydis*, which can capture variations in the biomass composition with respect to substrate composition. The flux analysis clearly shows that nitrogen-free compounds such as IA are expressed by an overflow metabolism as soon as a nitrogen limitation occurs. However, additional measurements are required to improve the model and to strengthen its predictive quality. Especially the parameters in the energy balance are rather uncertain, but at the same time, they have a strong influence on the optimization results. The next steps require the measurement of all components in the fermentation broth to determine the distribution of by-products. In addition, the elemental biomass composition must be analysed to determine its dependency on the nitrogen supply. Furthermore, it would be very attractive to measure metabolic fluxes within the cell to understand the relative contributions of the possible IA synthesis pathways. Such an experimental programme combined with model structure adjustments and parameter calibration would provide important hints for the genetic improvement. For example, undesired by-products such as glycolipids can be eliminated, if their synthesis pathways could be disrupted, in order to optimize *U. maydis* for the production of IA.

Furthermore, the (improved) metabolic model will be integrated in a fermenter model linking the substrate and product fluxes on the metabolic level to the concentrations in the fermenter broth (see, e.g., (Sainz et al., 2003) for a related approach). Such an integrated model could support the model-based design of experiments to first improve the predictive power of the model and then to determine optimal operating conditions for a continuous fermentation, which maximizes the overall yield of IA. The model would also be instrumental to support the design of future reactor configurations and downstream processing of the broth.

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