Summary

Different strategies for the optimisation of *Escherichia coli* as production organism were investigated using metabolic modelling tools. An overview of the state of the art of metabolic flux analysis (MFA) is given, starting with different metabolic model check methods (elemental composition check, dead-end detection, parallel pathway identification) and continuing with the general technique for solving overdetermined metabolic models and balancing the measured fluxes or eventually, in the case of flux data being inconsistent between each other, finding the wrong measurement with the vector comparison test.

A drawback of MFA is that it is only applicable to cultures in pseudo steady state. Therefore a novel technique, called dynamic MFA, is presented. Dynamic MFA transforms time series of transient experimental data to a form suitable for MFA. Dynamic MFA is based on taking the derivative of the concentration profile at each time instant of interest and using this derivative to calculate net fluxes, that are subsequently used in the MFA framework. However, taking the derivative increases the noise on the data, thus suitable data smoothing algorithms have to be used. Polynomial smoothing is proposed, but it has some difficulties and much manual tuning is needed. Still, acceptable results were obtained.

These mathematical techniques were then applied on different biologically interesting and industrially relevant cases.

E. coli strains genetically engineered to produce more shikimate (an interesting starting compound for a number of chemicals, *e.g.* oseltamivir phosphate, the active compound of Tamiflu®, a drug against influenza), has a higher shikimate yield when grown under carbon-abundant conditions (phosphor-limited cultivations) than under carbon-limiting conditions. Using MFA, it is shown that this is not due to a lower flux towards the aromatic pathway (shikimate is an intermediary compound of this pathway), but is caused by the increased excretion of other metabolites involved in the aromatic pathway. Also, having access to internal flux values for ATP hydrolysis at different growth rates for cultures under carbon- and phosphor-limitation, the maintenance parameters are calculated for both types of cultivations. It is suggested that *E. coli* cells under carbon-abundance have no growth-associated maintenance but are utilising the maximal possible amount of ATP and the non-growth-

associated maintenance is as high as the total maintenance requirements of cells grown under carbon-limiting conditions near the wash-out rate.

A typical problem of *E. coli* cultivations is the acetate overflow metabolism. Different genetic strategies were assessed to reduce this waste acetate production. When disabling the acetate pathway, less acetate was indeed produced, but more lactate was found in the reaction broth. The approach in which the more fundamental problem of insufficient carbon going from the glycolysis to the Krebs cycle is remedied by overexpressing *ppc*, encoding PEP carboxylase, gave better results. Obviously, the third approach in which both genetic strategies were combined, also performed well.

Next, it was assessed whether a *ppc* overexpressing mutant had improved capabilities for producing recombinant proteins. The model protein used was β-galactosidase. A plasmid containing *LacZ* (encoding β-galactosidase) with an IPTG inducible promoter, was introduced in wild-type cells and *ppc* overexpressing strains. For both, chemostats were run, and when steady state was attained, IPTG was added to the reactor broth, starting the recombinant protein production. A twelve times higher protein production flux was observed in the *ppc* mutant.

The intracellular flux distributions of carbon-limited continuous cultures switched to NH_3-limitation and *vice versa*, was investigated using dynamic MFA. When switching from nitrogen-limitation to nitrogen-abundance (carbon-limitation), a lag phase of several hours in which the growth of cells almost stopped, was observed. No clear reason could be given for this. However, the extended period of the lag phase (5 hours) suggests that the cause is probably genetic. So far, such lag phase has only been described in literature for *glnE* knock-out mutants. Gene *glnE* encodes ATase, the protein responsible for the regulation of glutamine synthetase. When, under nitrogen excess, ATase is not functioning anymore, the toxicity of NH_3 is due to accumulation of glutamine/glutamate. Such a lag phase was not observed in the case of carbon-limited cultures switched to nitrogen-limitation.

Extending the concept of chemostats, continuous cultures with oscillating environmental conditions were run. The oscillations were between aerobiosis and anaerobiosis. Two periods were chosen for the oscillations: 4 minutes and 30 minutes and in each case half of the period was aerobic, the other half being anaerobic. Gene expression profiles, using Affymetrix® microarrays, were generated at different time points in the oscillations and compared to the gene expression profiles of fully aerobic and anaerobic cultures. In the 30 minutes oscillating experiment, it was observed that cells adapted faster to aerobiosis than to anaerobiosis. In the experiment where the oscillation period was 4 minutes, the difference in gene expression profile between the aerobic phase and the anaerobic phase was minimal and the expression profile of both phases were more similar with the fully aerobic culture than the fully anaerobic one.

This work reviewed and extended the applicability of MFA to dynamic experiments. The usefulness of the methodologies presented were illustrated on different biologically interesting and industrially relevant case studies. Furthermore a novel kind of experiments was conducted: applying controlled environmental oscillation on chemostat cultures. This setup generated promising results.