

# Analysis and modelling of shikimic acid metabolism in *Escherichia coli*

by

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## Abstract

Shikimic acid is a building block for the anti-influeza drug GS-4104. Shikimic acid can be produced by e.g. genetically modified strains of *Escherichia coli*. When modifying an organism it is important to understand and investigate the entire cellular metabolism. In this work a stoichiometric flux model for *E. coli* with a particular emphasis on aromatic amino acid metabolism was constructed. The difference between a control strain and a modified strain was also investigated. As expected shikimic acid was produced in the modified strain and not in the control strain. Problems in the CO<sub>2</sub> and O<sub>2</sub> measurements and the existence of unknown metabolites in the modified strain made it impossible to test and to verify the model with the raw-data. However after an "ad hoc" conditioning, the model was tested on data from the control strain.

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## 1. INTRODUCTION

Amino acids are today of considerable commercial importance, and are used in a wide range of applications. From additives in food to raw material in the pharmaceutical industry. Many of the amino acids are produced in large quantities (1).

The three aromatic amino acids, phenylalanine, tyrosine, and tryptophan all originate from the shikimate pathway, which also includes a wide range of interesting molecules. One of them is L-shikimic acid, which is an intermediate in the pathway. It has recently been found that shikimate is a good building block for the anti-influenza drug GS-4104 (2,3). Earlier it has been isolated from the plant *Illicium* (4). However it can also be produced fermentatively, using genetically modified strains of *E. coli*. Organic synthesis would result in a racemic mixture and is thus no alternative.

In many scientific fields complex mathematical models have been developed and are today important tools. Sophisticated models control every day processes. In microbiological and biochemical areas the development has just started. For this purpose Metabolic Flux Analysis-technique (MFA) has been developed. By a few number of measurements it is possible to calculate many of the fluxes within a cell.

The objective of this work was to construct a stoichiometric matrix that could be used for MFA and to compare a modified, shikimic acid producing strain with a control strain.

## 2. METABOLISM OF *E. COLI*

*E. coli* is one of the most well known bacteria. It has the central parts of the catabolism in common with most other microorganisms, but the anabolic reactions differ significantly between microorganisms. A complete description of metabolism can be found in F.C. Neidharts book "*Escherichia coli and Salmonella*" (5).

The shikimate pathway has been extensively investigated by J.W. Frost (6). This has led to a modified strains capable of both producing shikimate and quinate (6). It holds overexpressed genes for *aroF*, *aroE* and *aroB* and deletions of *aroL* and *aroK* leading to a stop of metabolism after shikimate.

The modified strain used in this work holds fewer modifications. Only the *aroL* is removed and a feedback resistant version of *aroF* is overexpressed.

## 3. METABOLIC FLUX ANALYSIS

Metabolic flux analysis, MFA, is based on flux balancing, which can be said to be a slight variation of mass balancing. The model is typically used for cells growing in a chemostat. This because of the desire for steady-state conditions.

For each metabolite ( $X$ ) in a metabolic system a flux balance can be written. Collecting all reactants and reactions in matrices will result in:

$$\mathbf{q} = \mathbf{T}^t \mathbf{r} \mathbf{x} \quad (1)$$

$\mathbf{q}$  is a vector with the value of *net volumetric rate of formation* for every compound used in the system. The vector  $\mathbf{r}$  contains the rates (fluxes) of all reactions and is normally unknown,  $x$  is the biomass concentration. The stoichiometric matrix  $\mathbf{T}$  holds all stoichiometric coefficients for reactions used in the system. Each column corresponds to a compound and each row to a reaction. Only reactions of the type branch point to branch point are considered, thus sequential reactions have to be condensed to a single reaction.

MFA is based on the pseudo steady-state (pss) assumption, illustrated in Fig. 1. The use of pss means that metabolites like B, C and NADH in Fig. 1 have a net formation rate of zero. Otherwise there would be an accumulation of the intermediate inside the cell.

Fundamentally equation (1) has three solutions: underdetermined, overdetermined and determined. Only the determined system will be treated here.

### Determined system

In the ideal determined system the T-matrix is square, the number of reactants equals the number of reaction and the solution can easily be found. The most common case is an undetermined system that has become determined through measurements of some of the fluxes. The number of fluxes, which have to be measured, equals the difference the between of number of reactions and the reactants in pseudo steady state.

#### Solving the determined system

The  $\mathbf{q}$  vector in equation (1), the volumetric rates of formation, can be divided into three subvectors:

$$\mathbf{q} = \begin{bmatrix} \mathbf{q}_m \\ \mathbf{q}_c \\ \mathbf{0} \end{bmatrix} \quad (2)$$

The number of rows on  $\mathbf{q}_m$  equals the number of necessary measurements,  $m$  stands for measured, and  $c$  for calculated.  $\mathbf{q}_c$  contains the calculated volumetric formation rates and  $\mathbf{0}$  contains only zeros since it represents those reactants assumed to be in pseudo steady state.

A similar distribution can be made for both  $\mathbf{T}^t$  and  $\mathbf{r}$  in equation (2) and will result in:

$$\begin{bmatrix} \mathbf{q}_m \\ \mathbf{q}_c \\ \mathbf{0} \end{bmatrix} = \begin{bmatrix} \mathbf{T}_1 & \mathbf{T}_2 \\ \mathbf{T}_3 & \mathbf{T}_4 \\ \mathbf{T}_5 & \mathbf{T}_6 \end{bmatrix} \begin{bmatrix} \mathbf{r}_m x \\ \mathbf{r}_c x \end{bmatrix} \quad (3)$$

The dimensions of the submatrixes can easily be found;  $a$  is the total number of metabolic compounds,  $b$  is the total number of reactions/fluxes and  $d$  is the number of compounds in pseudo steady

state. The sizes of the matrices are summarised in table 1.

**Table 1.** Dimensions of sub-matrixes.

Submatrix	Dimension
$\mathbf{T}_1$	$(b-d) \times (b-d)$
$\mathbf{T}_2$	$(b-d) \times d$
$\mathbf{T}_3$	$(a-b) \times (b-d)$
$\mathbf{T}_4$	$(b-a) \times d$
$\mathbf{T}_5$	$(b-d) \times d$
$\mathbf{T}_6$	$d \times d$

Use of linear algebra gives the following solutions for  $\mathbf{r}_m$ ,  $\mathbf{r}_c$  and  $\mathbf{q}_c$ . For further information the book "Bioreaction engineering principles" by J. Nielsen and J. Villadsen (14) should be consulted.

$$\mathbf{r}_m x = \mathbf{T}_7^{-1} \mathbf{q}_m \quad (4)$$

$$\mathbf{r}_c = -\mathbf{T}_6^{-1} \mathbf{T}_5 \mathbf{r}_m \quad (5)$$

$$\mathbf{q}_c = (\mathbf{T}_3 - \mathbf{T}_4 \mathbf{T}_6^{-1} \mathbf{T}_5) \mathbf{T}_7^{-1} \mathbf{q}_m \quad (6)$$

$$\mathbf{T}_7 = \mathbf{T}_1 - \mathbf{T}_2 \mathbf{T}_6^{-1} \mathbf{T}_5 \quad (7)$$

The properties of the submatrixes are very important. The  $\mathbf{T}_6$ -matrix must be non-singular and thereby invertable.

By using only branch point reactions in  $\mathbf{T}^t$  and thereafter by interchanging the columns it can be possible to find a satisfying  $\mathbf{T}_6$  matrix. This does not however guarantee the existence of a non-singular  $\mathbf{T}_7$ . Sometimes it helps to switch the rows on the upper part of  $\mathbf{T}^t$  and chose different measured metabolite formation.

## 4. CREATING A STOICHIOMETRIC MATRIX

The main source for the reactions used when setting up the stoichiometric matrix in the current work was Parmanik's and Keasling's article "Stoichiometric Model of Escherichia coli Metabolism..." (7). Additional sources for were Varma's and Palsson's "Stoichiometric flux balance models quantitatively..." (8), the CD-ROM "Escherichia coli and Salmonella" by F.C. Neidhart (5) and Ingraham *et al* "Growth of the bacterial cell" (9).

To rationalise the following assumptions were made:

- In the reactions where NADH and NADPH can be used interchangeably NADH is used under catabolic reactions and NADPH is used under anabolic reactions.
- FADH<sub>2</sub> can be and is replaced by NADH where it is used.

### Biomass formation reaction

The formation of biomass is a complex reaction and it can seldom be found in literature. To construct a biomass reaction tables found in Ingraham *et al* (9) were used together with the article by Varma and Palsson (8).

## Respiratory reaction

There are multiple parallel reactions for cellular respiration and the fraction of the total flow between them changes continuously. These reactions also have different stoichiometry as well as different cofactors. van de Walle and Shiloach (10) used in their metabolic flux analysis of a genetic modified *E. coli* the assumption of an ATP/oxygen-ratio of 1,33.

## The matrix

By starting with a large and singular matrix containing 95 reactions and 103 reactants and then removing part after part a smaller matrix could be constructed.

Only glycolysis, pentose phosphate pathway, citric acid cycle, and the shikimic pathway, leading to aromatic amino acids, were kept. Extra branch points were added for dehydroquinate, dehydroshikimate, and shikimate by adding excretion-reactions. Compounds that are phosphorylated are seldom excreted and unphosphorylated compounds are more easily excreted. In the shikimic acid pathway dehydroquinate and dehydroshikimate are unphosphorylated intermediates and therefore these were chosen for excretion. Totally the matrix now consisted of 32 reactions and 35 compounds. Reactions used can be found in the full report.

### 5. Materials and methods

## Fermentation methods

### Equipment used

Sterilisation was performed in a Varioklav typ EH from H+P Labortechnik GmbH. Fermentor used is a B. Braun Biotech International Biostat A with a maximum working volume of 2-liter. Gasflow was controlled by a BrokenHorst HI-TECH EL-FLOW® mass flow controller (MFC) and outgoing gas was analysed with a Adaptive Biosystems TanDem gasanalyser. pH-electrode and pO<sub>2</sub>-electrode came both from Mettler Toledo. pO<sub>2</sub>-electrode was of type InPro® 6000 O<sub>2</sub> series and pH-electrode was of the type Mettler Toledo combination pH Nr 405 DPAS-SC-K88/200. To collect data and control the MFC-device LabView® 5,0 from National Instruments was used. For spectrophotometric analysis a Spectrometric UNICAM Helios alpha was used together with the Vision 3,5 computer program from UNICAM. All centrifuges came from Hermel and models used were Z 513 K, Z 200 A and Z 160 M.

### Precultivation

Preculture was performed in 100 ml scale. Glucose concentration was 20 g/l. Concentration of minerals and other trace compounds are given in Appendix III. 1 ml of frozen cells was used to inoculate. Preculture was incubated for 24 h in baffled shake flasks.

### Batch cultivation

The glucose concentration was 10 g/l for batch cultivation in 2 litre scale. Concentration of minerals and other trace compounds are given in Appendix III. Aeration was 0,5 vvm and stirring was set to 1000 rpm. Inoculation was made with 30 ml from preculture.

### Chemostat cultivation

The batch start-up of chemostat was performed in a 1,5 litre scale and same glucose concentration as for normal batch mode.

Concentration of minerals and other trace compounds are given in Appendix III. Aeration was 0,5 vvm and stirring was 750 rpm. When almost all glucose in batch-phase was consumed pumping of media through the fermentor was started. Three different dilution rates were performed, approximately 0,08 h<sup>-1</sup>, 0,12 h<sup>-1</sup> and 0,2 h<sup>-1</sup>. Composition for the media pumped through the fermentor were the same as for media in batch-phase.

## Analytical methods

### Cell density and dry weight

Cell density was measured by optical density, OD, at 620 nm. In addition, cell dry weight was measured from a sample of 10 ml. The sample was washed twice with deionised water and centrifugation at 4000 rpm for 10 minutes. After drying in oven for 24 h sample weight was determined.

### Metabolites

Metabolite concentrations were analysed from a 1 ml sample. Before analysis with HPLC samples were thawed and centrifuged. Analysis of the following substrates and products were made: acetate, citrate, ethanol, formate, glucose (only for batch mode samples), pyruvate, lactate, and succinate. For HPLC-analysis the following equipment were used: Waters 1515 isocratic HPLC-pump, Water 2410 Refractive index detector with a temperature of 45-°C, Applied Biosystems 747 Absorbance Detector, Breeze version 3.20 software and a HPX 87H ion exclusion-column with the operating temperature 55 °C.

## 6. RESULTS AND DISCUSSION

Batch fermentations were performed to get a first characterisation of the bacteria used. Chemostat fermentations were performed to generate values to test the stoichiometric model.

### Batch cultivation

#### OTR and CER

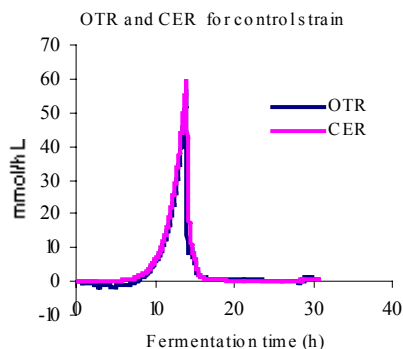
Measurements of the composition of out coming gas from the fermentor was used to calculate the oxygen transfer rate (OTR) that equals the consumption of oxygen and the carbon dioxide evolution rate (CER). Equation 8 was used to calculate both OTR and CER (11).

$$OTR = \frac{F \cdot 1000}{V} \cdot \left[ \left( \frac{C_{O_{2out}}}{100} \cdot \frac{C_{N_{2in}}}{C_{N_{2out}}} \right) - \left( \frac{C_{O_{2in}}}{100} \right) \right] \text{ mmol/l}^* \text{ h} \quad (8)$$

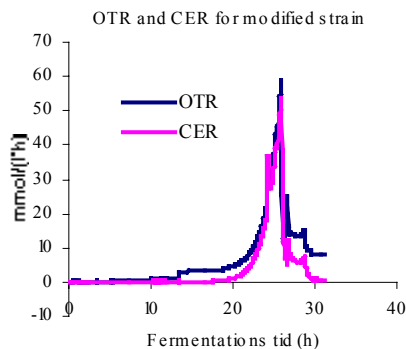
In Diagram 1 and Diagram 2 OTR and CER are plotted as function of cultivation time for the control strain and the modified strain respectively.

#### Growth curve

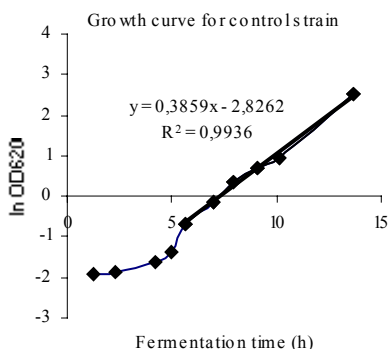
For the control strain the maximum growth rate was calculated to 0.4 h<sup>-1</sup>. For the mutant strain the maximum growth rate was calculated to 0.6 h<sup>-1</sup>, cf diagram 3 and 4. The difference in length of lag-phase could be seen here as well.



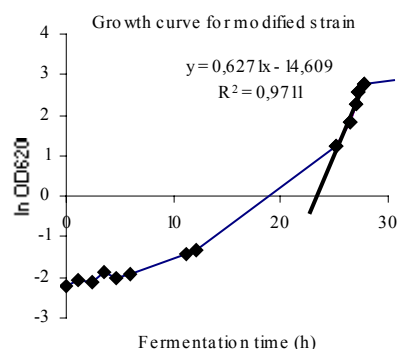
**Diagram 1.** OTR and CER for control strain.



**Diagram 2.** OTR and CER for modified strain.



**Diagram 3.** Growth curve for control strain.

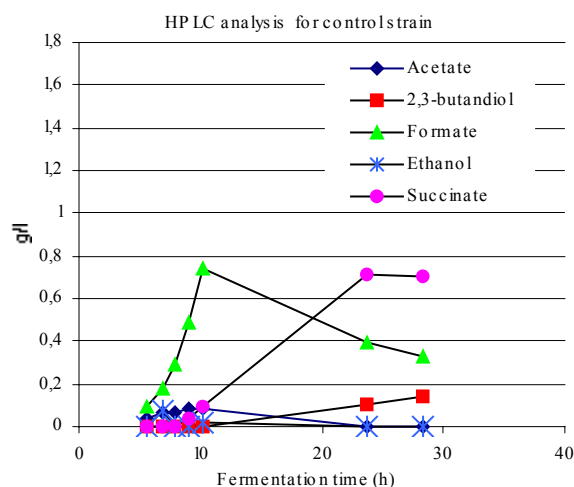


**Diagram 4.** Growth curve for modified strain.

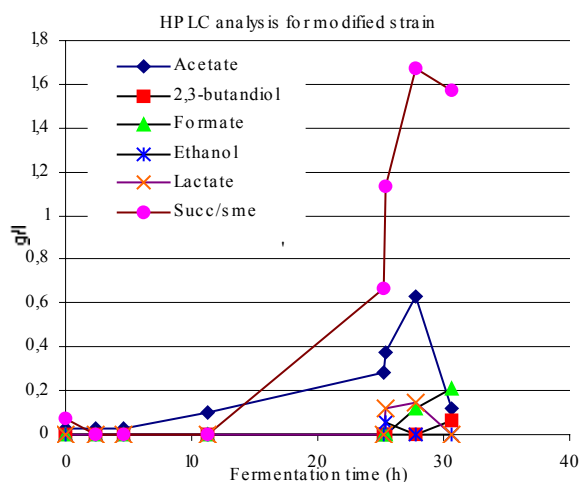
### HPLC-analysis

Only a minor number of metabolites were produced in detectable amounts. Some of the products are consumed in a late stage of fermentation. All acetate formed during fermentation was consumed in both strains. Formate was only consumed by the control strain to a certain extent and the modified strain produces formate where the control strain is consuming it. In Diagram 5 and 6 concentrations of different compounds in the fermentation media are plotted against fermentation time.

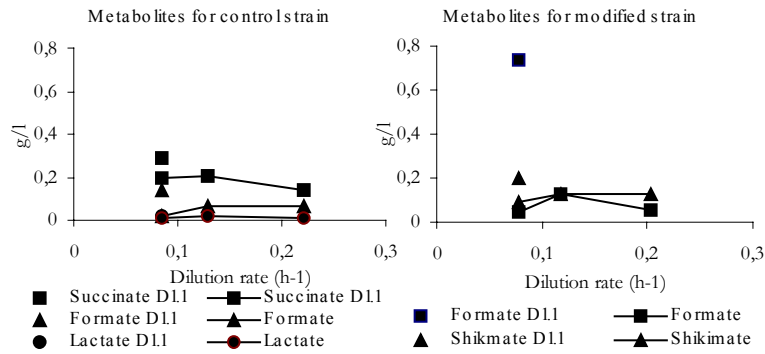
The desired shikimate was certainly produced to some extent although the amount was not quantified. Unfortunately, separation of succinate and shikimate could not be reached by the HPLC method used, but there is a significant difference in the area for succinate/shikimate for modified strain and the top for succinate for the control strain. This suggests that some shikimate has been produced.



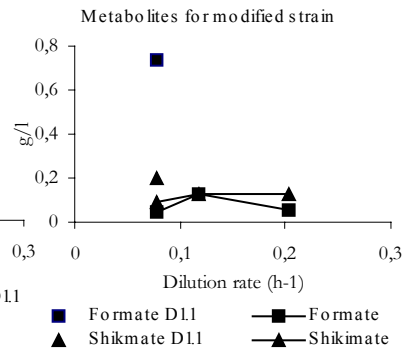
**Diagram 5.** Metabolites for control strain from HPLC-analysis.



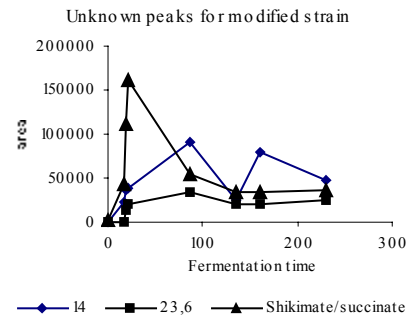
**Diagram 6.** Metabolites on batch\_modified from HPLC-analysis.



**Diagram 7.** Metabolites for control strain as function of dilution rate.



**Diagram 8.** Metabolites for modified strain as function of dilution rate.



**Diagram 9.** Unknown peaks and the peak for SUCC/SME for modified strain.

**Table 4.** OTR, CER and RQ-quotient for chemostat cultivations

Dilution rate (h <sup>-1</sup> )	Control strain			Mutant strain		
	OTR mmol/(l*h)	CER mmol/(l*h)	RQ	OTR mmol/(l*h)	CER mmol/(l*h)	RQ
D1.1	11	10	0,90	6,4	11	1,7
D1.2	13	11	0,85	8,5	11	1,3
D2	14	16	1,14	15	18	1,2
D3	26	26	1	17	23	1,4

**Table 5.** Dry weight and yield with respect to glucose for different dilution rates at steady state.

Dilution rate (h <sup>-1</sup> )	Control strain		Modified strain	
	Dry weight g/l	Yield g <sup>dw</sup> /g <sub>glucose</sub>	Dry weight g/l	Yield g <sup>dw</sup> /g <sub>glucose</sub>
D1.1	3.84	0.38	2.16	0.22
D1.2	3.645	0.36	1.88	0.19
D2	4.08	0.41	2.78	0.28
D3	4.31	0.43	2.25	0.23

### Dry weight

The final dry weight was larger for the mutated strain, and thus this strain had a greater biomass yield. Dry weight and yield can be found in Table 2.

**Table 2.** Dry weight and yield with respect to glucose at fermentation end.

Strain	Dry weight g/l	Yield g <sup>dw</sup> /g <sub>glucose</sub>
Control strain	3,40	0,34
Modified strain	5,43	0,54

### Chemostat cultivation

After batch start-up the cultivation started at the lowest dilution rate, and the dilution rate was then stepwise raised. After measurement of the highest dilution rate pumping speed was set to the lowest dilution rate again. Measurements from the first time at lowest dilution rate will be named D1.1 and second time at lowest dilution rate will be named D1.2. It should be noticed that the media used did not contain any of the trace component solution and therefore the modified strain was expected to grow slower than earlier. Dilution rates used are given in Table 3.

**Table 3.** Dilution rates for chemostats

	Dilution rate (h <sup>-1</sup> )	
	Control strain	Modified strain
D1.1, D1.2	0,084	0,077
D2	0,013	0,12
D3	0,22	0,20

### O<sub>2</sub> uptake and CO<sub>2</sub> evolution

As expected dilution rate affected both OTR and CER. In Table 4 these values are displayed together with the RQ-quotient. The RQ-quotient is defined as CER/OTR.

### Dry weight

As seen dry weight for the control strain increases with the dilution rate, i. e. the specific growth, cf. Table 5. The same could be expected for the modified strain but it cannot clearly be seen. Dry weight for the D2 is higher than for D1.1 and D1.2 but D3 is almost as low as D1.1 and D1.2. The control strain also had higher yield than the modified strain, this was expected since less carbon goes to byproducts in the control strain.

## Metabolites

Samples for HPLC-analysis were taken at steady state. In Diagram 7 and 8 concentration of metabolites are displayed as a function of dilution rate.

Two major unknown peaks were also found on the HPLC-chromatograms. Their size, in area units, on the RI-detector are plotted against fermentation time in Diagram 9, a major measurement error could be expected for the “14”-serie at fermentation time 135 h. Although it was not possible to identify the peaks, it was suspected that these two unknown peaks possibly could arise from dehydroquinone and dehydroshikimate. As these metabolites have almost the same molecular weight as shikimate the sizes of the peaks in the RI-detector are probably comparable to those of shikimate, see Diagram 9.

## Carbon and degree of reduction balance

### Control strain

HPLC-analysis showed that only a minor amount of products other than biomass and carbon dioxide were formed.

Neither the carbon balance nor the degree of reduction balance did close, and as much as 10-15% of the carbon went missing. This is too much and running the model on these data would be misleading. It is quite obvious that some kind of product and substrate is missing. To still be able to test the model an “ad hoc” data conditioning was used. Since no major unknown peaks were found on the HPLC-chromatograms for the control strain it was assumed that missing carbon was carbon dioxide. This compound does not affect the DR-balance so an additional assumption had to be made. Missing DR was assumed to be oxygen, the low RQ-value calculated suggest that the gas analyser has not worked properly. Measured values together with differences in balances and calculated values can be found in Table 6. The corrected RQ using modified values was reasonable.

### Modified strain

It was not expected that balances for the modified strain should close. The two major peaks in the HPLC-chromatograms should contain a lot of the carbon. The balances also showed this but there was a great variation in the result, see Table 7, in some of

the balances there was even a surplus of carbon. These results indicate major measurement

**Table 7.**  $\Delta C$  and  $\Delta DR$  for modified strain.

Dilution rate	$\Delta C$	$\Delta DR$
D1.1	-0,21	-1,6
D1.2	-0,36	-1,7
D2	0,039	-0,27
D3	0,18	-0,23

uncertainties and therefore a simulation of the shikimic acid producing strain was not possible using values from analysis made.

## Solving steady-state fluxes

Simulation of metabolism was performed for the control strain. Compounds in the vector  $q_m$  were: glucose, carbon dioxide, shikimate, oxygen, biomass, formate, succinate, lactate, dehydroquinone, dehydroshikimate, all with the unit  $\text{mol}/\text{g}_\text{h}$ . The flux distribution for D1.1 is given in Fig. 1.

As seen the problem point is fluxes around pyruvate and acetyl-CoA. It is calculated for all dilution rates an uptake of acetate and excretion of ethanol.

Measurements have not given any ethanol or in the media. This effect probably arose from earlier assumptions to close the carbon balance.

## DISCUSSION

### Batch cultivation

The most striking difference between the control strain and the modified strain is the length in lag-phase.

The maximum specific growth rate is also a point of difference. Here the expected relation between the modified and the control strain is the opposite. The modified strain has a maximum specific growth rate of  $0,6 \text{ h}^{-1}$  and the control strain only grows with a maximum specific growth rate of  $0,4 \text{ h}^{-1}$ . Normally modified strains grow slower than control strains. The explanation for this behaviour is probably the cultivation media. This since it contains a “trace compound solution” containing p-hydroxybenzoate,

**Table 6.** Carbon and degree of reduction balance for chemostat\_control.

Dilution rate	Measured values		Balance difference		Calculated values		
	OTR $\text{mmol}/\text{g}_\text{h}$	CER $\text{mmol}/\text{g}_\text{h}$	$\Delta C$	$\Delta DR$	OTR $\text{mmol}/\text{g}_\text{h}$	CER $\text{mmol}/\text{g}_\text{h}$	RQ
D1.1	11	10	0,18	-0,51	15	15	1,03
D1.2	13	11	0,20	-0,42	16	17	1,04
D2	14	16	0,14	-0,51	20	22	1,04
D3	26	27	0,13	-0,52	34	36	1,05

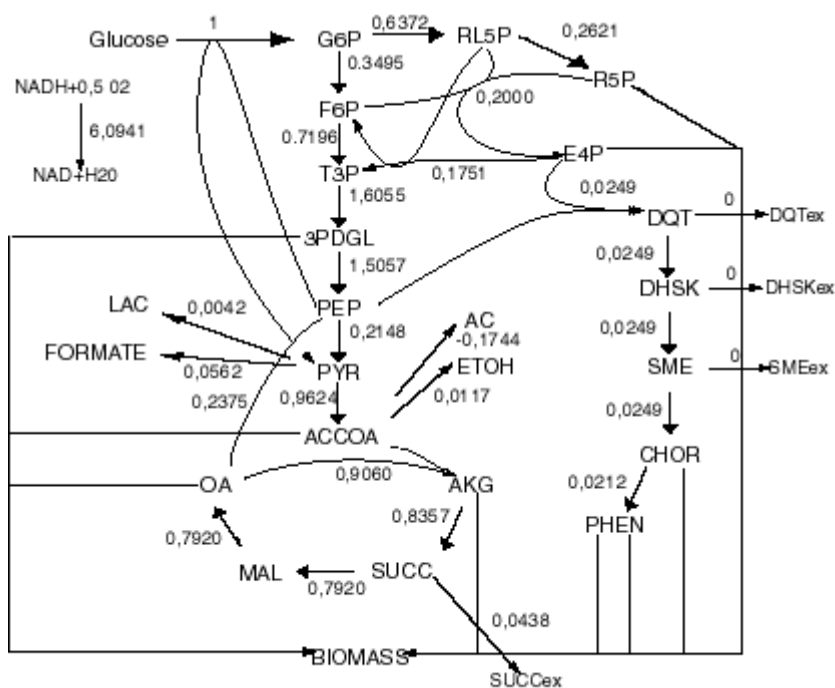


Fig. 1. Metabolic flux distribution calculated by the model.

p-aminobenzoate and 2,3-dihydroxybenzoic acid. These three compounds all participate in biosynthesis of macromolecules downstream of the shikimate pathway; p-hydroxybenzoate in the biosynthesis of ubiquinone, p-aminobenzoate in folic acid synthesis, and 2,3-dihydroxybenzoic acid in synthesis of enterobactin. They do not possess any direct regulation capabilities of the genes in the shikimate pathway but may have an effect on the overall regulation of the shikimate pathway.

### Chemostat cultivation

During chemostat cultivation the modified strain showed that it was capable of producing shikimate, especially during batch start-up, but apparently it produced substantial amounts of a by-product, possibly both dehydroquinone and dehydroshikimate. This could, however, not be proved since standards of dehydroquinone and dehydroshikimate were not accessible.

At the end of the chemostat run it had almost lost all its capabilities of producing shikimic acid. Since *aroF* is placed on a plasmidvector, which is present in a number of copies, the reasonable explanation for the difference between D1.1 and D1.2 could be the lowering of the copy-number. But this might not be the only answer as the control strain was not able to return to the same steady-state after disturbance.

### Modelling

It is difficult to comment on the functionality of the metabolic model at this time. Unfortunately, the carbon balances did not close satisfactorily. This could be due to inaccurate measurements, or production of unknown compounds. To be able to perform a better test additional analyses have to be

made and the carbon balance for the black box system has to close. One very critical point is e.g. the biomass composition.

## CONCLUSIONS

A stoichiometric matrix for the shikimate metabolism in *E. coli* was constructed from known metabolic pathways. It contains 32 metabolic fluxes and 35 reactants. The stoichiometric matrix was non-singular and could be arranged in a suitable way to be used in metabolic flux analysis. The biomass reaction in the model must, however, be adapted to fit the measurements of cellular composition after each batch to achieve a satisfying metabolic flux analysis.

With measurements made during this work there is no possibility to state anything about the functionality of the model. To do so the following things has to be done:

- Identification of unknown metabolites.
- Determination of the cellular composition.
- More accurate gas measurements.

The modified strain produced shikimate but the stability of modifications made were not as good as they could be. This problem could be avoided by the use of another stronger promoter on the plasmid or with chromosomal integration. Until better stability has been achieved it is suitable not to perform long fermentations. Only one steady state point should be investigated in the same chemostat cultivation.

It was also shown that the addition of "trace compound solution" to the cultivation media had a great impact on the specific growth rate. This could be good to keep in mind when designing and preparing cultivation media.

## ACKNOWLEDGEMENT

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