1. GENERAL INTRODUCTION

1.1. General motivation of flux quantification in organisms

Within the last decades, the demand for fossil resources such as crude oil and natural gas increased significantly. The growing utilization as energy source, fuel or as substrate for chemical production plays an essential role in depletion of these finite resources. Therefore, a shift from fossil to renewable resources, or in other words from a crude oil-based to a bioeconomy, is pursued. The usage of biological resources displays one possibility. These biological resources can be either biological material itself or products manufactured by the use of biological hosts in biotechnological processes. A broad range of substances which are currently manufactured based on crude oil can be produced biotechnologically. This includes bio fuels, pharmaceutically valuable substrates or fine chemicals [1-3].

Changing the metabolic network of an organism in order to enhance the flux towards a product of choice is of great interest. The development is supported by metabolic engineering with targeted genetic modifications for manipulating microbial hosts. Modification of an organism can be carried out more targeted and effective if the intracellular processes of the cell are known in detail. Obtaining information about intracellular processes can be beneficial for both the general understanding of an organism, as well as verification of changes introduced by metabolic engineering. The phenotype of the manipulated organism can be compared to the phenotype of the unaffected organism and the efficiency of the engineered organism can be investigated.

The information to be compared in the assessment of the intracellular processes describes the use of individual intracellular reaction rates (fluxes) within the cell. These fluxes are finally quantified in order to compare the unaffected and manipulated organism. In organisms with linear metabolic pathways, the intracellular carbon fluxes can simply be quantified by balancing substrate uptake and product formations rates. Simple metabolic networks like in *Lactococcus lactis* (Figure 1a), can therefore be investigated under the assumption of stationary conditions, meaning constant metabolite pool sizes within the investigated time span [4,5].

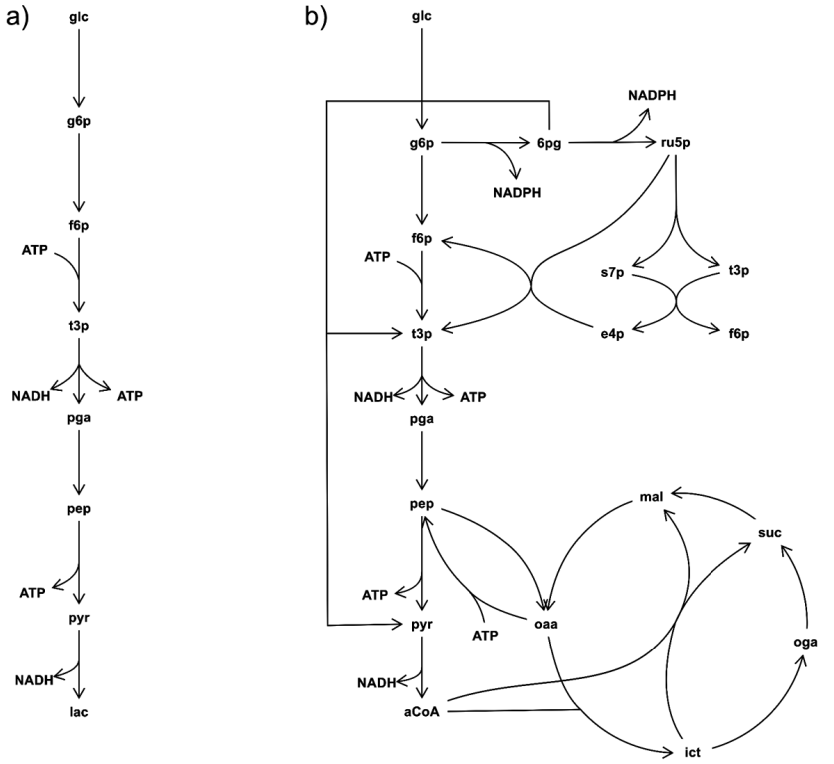

Figure 1: Central carbon metabolism of Lactococcus lactis *(a) and* Escherichia coli *(b) during growth on glucose adapted from [5].*

In organisms with parallel, branched, and cyclic metabolic pathways not directly connected to fluxes that can be measured, like in *Escherichia coli* (Figure 1b), a stoichiometric approach for quantification of carbon fluxes is insufficient due to the high degree of freedom. Hence, these equation systems are composed of too few equations with too many unknowns wherefore the number of equations has to be enhanced. ^{13}C-based metabolic flux analysis (^{13}C-MFA) is used for enhancing the number of equations and allows the deciphering of those. This is achieved by balancing unresolvable fluxes integrating additional constraints from stable isotope data reflecting information about carbon utilization within the metabolic network [4-6].

1.2. ^{13}C-metabolic flux analysis

The method of ^{13}C-MFA has been developed as an extension of stoichiometric flux analysis. Due to the investigation of carbon utilization, complex networks with parallel and cyclic

pathways can be resolved. In ^{13}C-MFA, the cells are fed with a substrate labeled with stable ^{13}C-isotopes at predetermined positions. Typically used ^{13}C-isotope labeled substrates are uniformly labeled U-^{13}C-glucose or 1-^{13}C-glucose [7,8]. Alternative ^{13}C-isotope labeled substrates such as acetate, lactate, and methanol have already been reported in several studies [9,10]. Since the labeled substrates are taken up by the organism, they are incorporated into metabolites and biomass. Depending on the metabolic pathways used for the formation of metabolites, differences in the ^{13}C-isotope distribution can be observed within the metabolites [11]. The cells are harvested and subsequently extracted or hydrolyzed, whereby the metabolites of interest become detectable. These metabolites are measured and their ^{13}C-labeling is detected. In combination with growth data such as biomass formation and substrate uptake rates, these labeling data are used for computationally solving the equation system.

These computations can be carried out with the support of various software programs such as OpenFLUX [12], 13CFLUX2 [13], Metran or FiatFlux [14]. These programs differ regarding the underlying algorithm [15]. In OpenFLUX, 13CFLUX2 and Metran global iterative fitting is used for metabolic flux calculation. ^{13}C-labeling patterns of measured metabolites and extracellular rates such as uptake and production rates are added as constraints by the user. Based on a model of the cellular carbon metabolism, semi-random flux distributions are used for simulation of theoretical ^{13}C-labeling patterns and extracellular rates. This procedure is repeated, starting from the previously calculated parameters until the deviation between the simulated and measured data is reaching a minimum. The whole metabolic network is used for flux calculation, directly resulting in absolute flux values for every flux within the model.

The flux calculation approach used in FiatFlux is the so called flux ratio analysis [14]. In contrast to global iterative fitting, the flux ratio analysis only focusses on selected fluxes affected by different pathways and therefore local labeling changes are considered. In this case, the labeling data of selected metabolites are used for calculating the relative amount this metabolite is formed via given pathways. Extracellular rates are not needed for determination of flux ratios (Figure 2) [8].