Fusing prior knowledge with microbial metabolomics

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Chapter 1

General introduction

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1. General introduction

1.1 Metabolism

Metabolism \[1\] comprises the whole set of biochemical reactions that occur in the cells of living organisms to sustain life. These reactions allow for uptake of nutrients from the environment and conversion into energy and building blocks necessary for organisms to grow, reproduce, maintain their structures, and respond to their environments. Metabolism can be divided into two categories. Catabolism is the set of reactions that break down large molecules such as polysaccharides, lipids, nucleic acids and proteins into smaller units such as monosaccharides, fatty acids, nucleotides, and amino acids, respectively and release energy. Whereas the opposite of catabolism, which is named anabolism, comprises the set of reactions for construction of molecules from smaller units under requirement of energy. Substrates, intermediates and end-products of metabolism are typically low molecular weight organic compounds, known as metabolites. The step-by-step modification of metabolites in living cells occurs through series of biochemical reactions (denoted as metabolic pathways), most of which are catalyzed by proteins (enzymes) that are encoded on the genome (an organism’s hereditary information). Because intermediates and end-products of one biochemical reaction can be the substrate of another, metabolic pathways form extensive networks, which are referred to as metabolic networks (see Figure 1.1). Metabolites form, so to say, the links that connect pathways in the metabolic network.

1.1.1 Metabolism: fluxes and concentrations.

There are many fields of research that study metabolism and use information about metabolic networks and its pathways. For example in cellular systems biology the behaviour of metabolism in the context of cell growth is studied in terms of metabolic fluxes \[2,3\]. Another example is metabolic engineering, which is defined as the directed improvement of cellular properties through the modification of specific biochemical reactions or the introduction of new ones with the use of recombinant DNA technology. To achieve this goal metabolic engineering does not focus on individual enzymatic reactions but interactions of biochemical reactions and metabolic pathways in a metabolic network with emphasis on metabolic fluxes and their control under in vivo conditions \[4\].

The metabolic flux can be defined as the rate at which material is processed through a metabolic pathway \[5\]. The flux is a fundamental determinant of cell physiology and a critical parameter of a metabolic pathway. Along with intracellular metabolite concentrations, fluxes define the information used for capturing metabolism and cell physiology in a certain environmental condition. Fluxes also determine the degree of engagement of various enzymes in a conversion process. However, engagement does not state anything about activity, for enzymes may be present and active yet carry very little flux. The determined pathway flux defines the extent to which pathway enzymes participate in a conversion process.
1.1.2 Metabolomics

Metabolomics, as one of the most recent members in functional genomics, has become an accepted and valuable tool in life sciences for studying metabolism. It deals with the identification, qualitative and quantitative (in terms of concentra-
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measurements of all metabolites in a sample in a specified biological state. The total set of metabolites has been termed the metabolome [8] and is a reflection of the phenotype of the system under the studied conditions [9]. Metabolomics studies are set up around a biological question with the main goal to gain new insights into biochemical processes and pathways, and to relate these to characteristic features at the other functional genomics levels (genomics, transcriptomics and proteomics), the physiological and phenotypic level. An important step in the pipeline or workflow [10, 11] of a metabolomics study is to set up experiments and generate data. The center of attention in this thesis is microbial metabolomics, so metabolomics data that finds its origin in micro-organisms.

1.2 Fermentation

For microbial metabolomics studies fermentations form the experiments from which samples are taken to acquire data necessary for the study. To ensure that enough variation is induced and captured relevant to the biological question for which the study was set up, fermentations are often performed according to an experimental design. Examples of fermentations according to a full factorial design are given in [12, 13]. Fermentation is a very ambiguous term, as over time it got different meanings to biochemists, microbiologists and industrial microbiologists. Industrial microbiologists use the term fermentation to describe any process for making products that are useful for humans, through the intentional use of mass cultures of micro-organisms [14], such as bacteria and fungi. Fermented products find their application as food, as well as in general industry. Food products such as bread, beer, wine, cheese, curds and yoghurt come to mind and these products have been produced this way for thousands of years, long before mankind had any knowledge of the micro-organisms involved. Industry focuses on products that have economic value, such as pharmaceutical and medical compounds (e.g. antibiotics, hormones, steroids), solvents, organic acids, chemical feedstocks, amino acids, and enzymes. There are five major groups of commercially important fermentations: (i) those that produce microbial cells (or biomass) as the product (e.g. yeast), (ii) those that produce microbial enzymes, (iii) those that produce microbial metabolites, (iv) those that produce recombinant products, and (v) those that modify a compound which is added to the fermentation (the transformation process).

Fermentations are performed in bioreactors [15], as shown in Figure 1.2, ranging in size from laboratory grade to industrial. Bioreactors can be operated in different modes: (i) batch, where the fermentation proceeds without addition of fresh growth medium; (ii) fed-batch, where nutrients (in the form of growth medium) are added incrementally at various times during the fermentation; no growth medium is removed until the end of the process; (iii) continuous, where fresh growth medium is added continuously during fermentation, but there is also concomitant removal of an equal volume of spent medium containing suspended micro-organisms. An example of a continuous bioreactor is the chemostat [16, 17], in which the growth rate of the micro-organism can be easily controlled by changing the rate with which medium is added to the bioreactor. Batch operation is the most common laboratory
1.2. Fermentation

Figure 1.2: General design of a bioreactor

Figure 1.3: Phases of bacterial growth. Source: Medical Illustrations by Michał Komorniczak (PL)
growth method in which bacterial growth is studied and also a commonly used mode for microbial metabolomics studies, but it is only one of many.

1.2.1 Microbial growth during batch fermentation

The growth of a microbial culture during a batch fermentation can be divided into a number of stages as is depicted in Figure 1.3. At first a particular micro-organism needs to be introduced into the selected growth medium, the medium is said to be inoculated with the particular micro-organism. Growth of the inoculum does not appear to occur immediately, there is the period of adaptation, referred to as the lag phase. Following the lag phase, the rate of growth of the micro-organism steadily increases until the cells grow at a constant, maximum rate. This period is called the log, or exponential, phase. The rate of growth slows down after a certain time of exponential phase, due to the continuously falling concentrations of nutrients and/or a continuously increasing (accumulating) concentrations of toxic substances excreted by the micro-organism into the medium. Eventually, growth ceases and the microbes enter the so-called stationary phase. Within the stationary phase the biomass remains constant, except when certain accumulated chemicals in the culture lyse the cells (chemolysis). After a further period of time the viable cell number declines, when the culture enters the death phase.

As well as the kinetic description of growth, given in the previous paragraph, the behaviour of a culture may also be described according to the products which it produces during the various stages of the growth curve. During the log phase of growth the products produced are essential to the growth, development and reproduction of the cells of the micro-organism and include amino acids, nucleotides, proteins, nucleic acids, lipids, carbohydrates, etc. These products are referred to as the primary metabolites and many of them are of considerable economic importance and are in industry produced by means of fermentation. During the stationary phase some microbial cultures synthesize compounds that are not essential for growth or survival of the producing organism, however this is not true for all types of microbes. These compounds are referred to as the secondary compounds of metabolism, also called secondary metabolites. The production of these metabolites is tightly regulated and dependent on the immediate environment. Although secondary metabolites are not directly related to growth, development and reproduction these compounds do find their origin in intermediates of primary metabolism. The group of secondary metabolites includes both simple molecules such as alcohols, sugars and organic acids; and complex compounds such as polyketides, flavonoids, terpenes and non-ribosomal peptide compounds. They show a huge functional diversity, including functional classes such as antibiotics, pigments (photoprotection), hormones/pheromones, cytostatics, systemic toxins (phytotoxins, fungicides, insecticides and immunosuppressives) and many other and are like primary metabolites also of great interest for industry.
1.3. Metabolome analysis

Once fermentation samples have been processed (extraction, addition of standards, etc.) to a state ready for measurement of the metabolome a metabolite analysis strategy has to be chosen. Different strategies \[9,23–25\] exist depending on coverage and quantitation (compound identity, sensitivity, accuracy):

1. **Metabolite target analysis**: Quantitative analysis of one or a few metabolites of interest, ignoring all the non-target peaks present in the sample.

2. **Metabolite/metabolic profiling**: Analysis for identification and approximate quantification of a group of metabolites related by similar physical and chemical properties or associated to specific metabolic pathways.

3. **Metabolic fingerprinting**: Global, rapid and high-throughput analysis of samples without identification and quantification of metabolites for purpose of classification or screening.

Going from targeted analysis to fingerprinting the data quality decreases, whereas the number of metabolites considered greatly increases \[26\]. Metabolomics, as defined in section 1.1.2 can be placed as analysis strategy being one step further than metabolic profiling \[9\,27\]: instead of aiming to obtain an inventory and approximate quantification of a group of metabolites present in a sample, it aims at identifying and quantifying the full metabolome. Not one analytical technique, or combination of techniques, can currently determine the full metabolome in samples for the simple reason that the metabolome comprises too many different compound classes for one technique to handle and not all metabolites are known to date. Because of this complexity of the metabolome it has been suggested that the term metabolomics should not be used as an analytical subsection but should rather be taken as a scientific keyword \[28\,29\]. Metabolomics, therefore usually consist of a combination of aforementioned analysis strategies. Identification of metabolites is sometimes performed after data analysis \[11\], which can pinpoint the most relevant compounds (biomarkers).
Apart from analysis strategies focusing on intra-cellular metabolites, there is also one called metabolic footprinting [30]. It is the high-throughput classification by global measurement of metabolites secreted from the intra-cellular volume into the extra-cellular spent growth medium, referred to as the exo-metabolome [31]. Metabolites in the intra-cellular volume are in this context referred to as the endo-metabolome. Metabolic footprinting represents a niche within metabolomics, because of its focus on the analysis of the exo-metabolome. Although metabolic footprinting represents only a fraction of the entire metabolome, it provides important information for functional genomics and strain characterization [32]. Information about the exo-metabolome could provide a key to understanding cell communication mechanisms and aid metabolic engineering of industrial biotechnological processes. Due to the strong and intertwined relationship between intracellular metabolism and metabolic footprinting, metabolic footprinting can provide precious information about the intracellular metabolic status and assist in further interpretation of metabolic networks and metabolic fluxes.

1.3.1 Analysis techniques

As mentioned in the previous section the metabolome consists of many different classes of compounds and multiple analysis strategies are employed with the ultimate goal of analysing a large fraction or all of the metabolites. Therefore, a range of analytical techniques and not a single one needs to be employed to analyse the metabolome [33, 34].

Two analytical techniques commonly associated with metabolome analyses using the fingerprinting strategy are Nuclear Magnetic Resonance (NMR) [35–37] and Mass Spectrometry (MS) [28, 38, 39]. Both are capable of handling a wide range of metabolites in a single measurement without pre-selection of specific analytes. These technologies allow both the identification and quantification of metabolites, meaning they are also applicable with other strategies. The main advantage of NMR is that it does not require physical or chemical treatment and is non-destructive, therefore, allowing samples to subsequently be analysed with another technique. On the other hand MS is more sensitive than NMR.

Many variations of MS exist, but the more traditional approach is coupling MS as detection method to chromatographic techniques, e.g. gas chromatography (GC) and liquid chromatography (LC). GC-MS [40] provides a very sensitive technique, however is limited to small compounds that are thermally stable, volatile, or can be made chemically volatile by means of derivatization. Contrary to GC-MS, LC-MS [41] allows for the separation and characterization of the majority of metabolites including different groups of compounds, hydrophilic as well as hydrophobic, salts, acids, bases, etc. The separation of each group in LC-MS is dictated by the properties of the metabolite, which determines which column type (stationary phase) and mobile phase to use for successful separation [42]. This makes LC-MS a powerful tool for metabolomic studies. For microbial metabolomics GC-MS and LC-MS are analysis techniques that are commonly used for identification and quantification of intra-cellular metabolites.
1.4 Analysis of metabolomics data

1.3.2 Post measurement processing

Once samples have been measured the raw instrumental data needs to be processed into extracted data (peak tables consisting of identified metabolites and their concentration in the measured samples). Raw data from chromatographic instruments hyphenated with MS-detection like GC-MS and LC-MS, as mentioned in the previous section, is two-dimensional in nature consisting of intensities for chromatographic retention times ($r_t$) and mass to charge ratios ($m/z$). It becomes three-dimensional when multiple samples have been measured, which adds a mode to the collected raw data. Processing this type of data should take into account and/or correct:

- baseline drift with respect to $r_t$.
- peak alignment with respect to $r_t$ and $m/z$ in order to line up peaks so that chromatographic shifts are reduced to a minimum and variations caused by column ageing and column cuts would be eliminated.
- detector response with respect to the measured intensities of known control samples.
- noise and artefacts by the measurement instrument by setting thresholds.

When analysing complex mixtures like metabolomes, elution of two or more metabolites with the same $r_t$ is encountered commonly and peaks will overlap [43, 44]. This severely complicates detection and quantification. Deconvolution [45] is the most promising strategy to extract pure metabolite signals from the data. It is based on the idea that a raw signal is a superposition of multiple single-metabolite responses. The underlying MS spectra and chromatograms can be obtained by imposing a model of single-metabolite contributions on the raw signal. The derived pure chromatograms can subsequently be used to calculate relative concentrations and the pure MS spectra for metabolite identification by matching the spectra against reference libraries. Reference libraries exist for GC-MS [46] but are far from covering all metabolites. Reference libraries for LC-MS, however, pose a problem because of the large diversity of compounds measured, large variations in fragmentation patterns for different types of instruments and low between instrument reproducibility. The most common strategy for quantification in LC-MS for that reason is peak-picking. Identification and quantification is labour intensive process, that generally takes much longer than the measurement the samples. For absolute quantification of metabolite concentrations internal standards are required. Often measurements remain intensities due to the lack of proper standards and the requirement for too many standards.

1.4 Analysis of metabolomics data

Data analysis is of crucial importance to metabolomics studies after post measurement processing, since it is the way to extract valuable information from the processed data (peak tables) that can answer the biological question for which the
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study was set up. For microbial metabolomics typical research questions are among others: (1) finding bottlenecks in the production of desired products for strain optimization [13], (2) optimization of the growth medium to improve product yield, (3) identification of product onset, (4) identification of differences between wild type and overproducing strains, (5) characterization of mutant strains [47], (6) finding metabolites that are related to desired product formation in order to unveil the metabolic pathway involved, (7) predicting the effect of quality differences of different batches of complex media on productivity, and (8) identification of metabolite dependent regulatory interactions.

1.4.1 Methods, data and their challenges

The methods used for analysis of microbial metabolomics data are similar or equal to methods from chemometrics [48, 49], which deals with the analysis of data obtained from experiments in chemistry. Those methods can roughly be divided into two categories: (i) methods directed at identifying differences between samples from different groups with a clear goal of prediction for new samples, these are so-called supervised methods and contain regression as well as classification methods (ii) methods directed at detecting patterns by data exploration without a prediction goal, which are so-called unsupervised methods.

Methods from the first category that are commonly applied to microbial metabolomics data are Partial Least Squares (PLS) [50], Partial Least Squares Discriminant Analysis (PLS-DA) [51]. A feature which is shared by these methods is that they use one or several biological properties of the data set, e.g. yield or information about the specific biological group the data belongs to like wild-type vs. mutant strain. Various modifications of PLS and PLS-DA exist, like the multiway generalization nPLS(-DA) [52, 53] and a recent modification called Orthogonal-PLS(-DA) [54, 55]. In OPLS(-DA) only the part of the processed data that is linearly related to the regression/classification problem is considered, with the intention to simplify the interpretation.

Exploratory methods for discovering patterns in highly multivariate data (second category as mentioned above) are often based on finding a low-dimensional representation of the data. Principal Component Analysis (PCA) [56] is such a method and can easily be called the workhorse of metabolomics data analysis, since it is the most applied method for exploring metabolomics data in general.

Each available method, whether it falls into the first or second category, has some underlying assumptions with respect to the data to be analysed. The most common assumptions of currently used methods are that the explored relationships between variables (metabolites) are linear in character and the model components needed for describing these relationships are independent of each other (orthogonal to each other). Another very important assumption is that the measurement of each metabolite is independent and identically-distributed (i.i.d). These assumptions with respect to microbial metabolomics data are hardly ever satisfied, which complicates data analysis and subsequent interpretation of analysis results. On the other hand microbial metabolomics data itself present multiple challenges which complicate data analysis and interpretation of analysis results:
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- it is high dimensional, more metabolites than samples are measured (so-called curse of dimensionality [57]) and metabolites are highly correlated, which can easily lead to overfitting.

- high noise levels, which can overwhelm biological phenomena present in the data.

- complex structure, where the data consist of contributions from several experimental design factors that are not separated during data analysis and can seriously hamper the interpretation.

- longitudinal character and the problem of synchronisation of data stemming from different fermentation batches.

- missing data due to instrument failure, non detects in the measurement or metabolite concentrations below the detection limit.

Also the fact that processed data often needs to be pretreated prior to data analysis provides a challenge. One pretreatment can enhance the results of a data analytical method and aid the interpretation of the analysis results, whereas it might obscure the results of another analysis method. The choice of pretreatment is, therefore, often restricted by the chosen data analysis method, which in turn depends on the biological question to be answered and the structure of the data to be analysed. Three classes of applied data pretreatment methods [58] are: (i) centering (ii) scaling and (iii) transformations. The most applied form of centering is mean centering, which converts all metabolite concentrations to fluctuations around zero instead of around the mean of the metabolite concentrations with the purpose of focussing on differences between samples. The effect of mean-centering with respect of metabolite concentrations is, however, that after performing it the obtained values lose their direct link to concentrations since now negative values also occur. The value 0 also gets a new meaning and no longer means not present or not important. Scaling of processed metabolomics data is performed by dividing each metabolite by a factor, the scaling factor, which is different for each metabolite. Various forms of scaling exist and have been described in detail with respect to metabolomics data in [58]. The purpose of scaling usually is to make metabolites comparable to their biological response or equalize their relative importance. Scaling of processed data prior to analysis should be done with care for it could result in noise amplification and cause an increase in heteroscedasticity of the data. Transformations are nonlinear conversions of processed data, e.g. the log transformation and the power transformation. They are generally applied to correct for heteroscedasticity [59], convert multiplicative into additive relations and adjust skewed distributions to make them (more) symmetric. As mentioned before currently applied methods assume relations between metabolites to be linear, however relations between metabolites in metabolomics data can also be multiplicative in nature. A transformation of the processed data and subsequent analysis with a method that assumes a linear relation between metabolites could then be used to identify these multiplicative relations between metabolites.
Which method to use from the large variety of available methods is not always clear, but the choice has to be such that it fits the research question and the structure of the data to be analysed. Choosing for example PCA when the goal is to identify metabolites that strongly relate to the yield of fermentations would not be logical, since this is typically a supervised regression problem. With respect to the structure of the data an example would be to choose a multi-way analysis method when the data consists of metabolite concentrations from samples taken over time from fermentations performed with different carbon sources.

1.4.2 Fusing Prior knowledge

As mentioned in section 1.4.1 the analysis of microbial metabolomics data and following interpretation suffers from inconsistencies regarding underlying assumptions of the commonly applied methods and challenges posed by the data itself. There are, therefore, two ways to deal with these problems: (1) adjust the data so it matches the assumptions of underlying the methods, and (2) adjust the methods so they can deal with the challenges the data present. Both adjustments can be guided by prior knowledge about the metabolomics study:

1. information available from previous experiments and from literature about the underlying (micro)biology with respect to the biological question for which the study was set up.
2. how the study was set up and the way experimental data was generated, e.g. the experimental design.
3. how the metabolomics data was collected and measured, e.g. where samples taken over time from a fermentation (longitudinal data) and/or is something known about the sampling/analytical measurement errors.

Development of methodology guided by prior knowledge to facilitate data analysis and interpretation has only recently been engaged and for that reason not many methods are available in the field of metabolomics. ANOVA-Simultaneous Component Analysis (ASCA) [60,61] is an example in which the underlying experimental design is used to partition contributions of different factors within the highly multivariate metabolomics data set. Another recent example that is more suited for incorporating (micro)biological prior knowledge is called Grey Component Analysis [62]. This exploratory method is based on PCA, but uses a soft penalty on the scores. The penalty allows the user (e.g. a microbiologist) to direct the scores to some predefined values, that represent the hard prior knowledge. The amount of emphasis on the penalty defines the confidence the user has in the prior knowledge being present, but also allows for a weighted average between data and prior knowledge.

1.5 Scope and outline of the thesis

This thesis, entitled “Fusing prior knowledge with microbial metabolomics”, deals with combining prior knowledge in microbial metabolomics. It not only focuses on
fusing prior knowledge with the data analytical aspect of a microbial metabolomics study, as described in section 1.4.2 but also on the use of prior knowledge within various aspects of such a study. Figure 1.4 schematically displays how the chapters in this thesis relate to the aspects of a microbial metabolomics study visualised in a metabolomics pipeline.

In a microbial metabolomics study the data for studying metabolism is obtained by sampling from fermentations followed by metabolome analysis of these samples. Data for studying metabolism of a micro-organism can, however, also be obtained in silico. In the construction of a genome-scale network of micro-organism prior knowledge is used about the genome sequence and gene-protein-reaction associations with respect to the organism obtained from databases, textbooks and other scientific publications. Within the framework of constraint-based modeling genome-scale networks of micro-organisms can be used to infer flux states of metabolism under different environmental conditions by means of in silico simulations. In Chapter 2 the genome-scale network of a lactic acid bacterium, named Lactococcus lactis MG1363, is used to generate flux distributions for multiple in silico environmental conditions, mimicking laboratory growth conditions. These flux distributions serve as raw data to investigate and pinpoint differences and similarities in metabolism between the various environmental conditions. Hence Chapter 2 exemplifies how prior knowledge can be used in the creation of data for studying metabolism.

As mentioned in section 1.4 scaling is often performed prior to data analysis, but has to be done with care for it might result in noise amplification and increase the heteroscedasticity of the data. Additionally methods commonly used for data analysis assume that each measured variable (metabolite) in the data set has the same probability distribution as the others and all are mutually independent (i.i.d.), however this is hardly ever the case for metabolomics data. Adjustment of the data guided by prior knowledge could deal with this problem and allow commonly used methods to be applied and possibly improve interpretation of the analysed data. Prior knowledge about the noise characteristics of the data is used in Chapter 3.
to adjust the processed raw measurement data. A procedure is introduced for multivariate data, that does not suffer from noise amplification by scaling. The procedure is used as a filter and can be used prior to any subsequent scaling and multivariate analysis of the data with commonly applied data analysis methods.

A large number of metabolites are measured in a microbial metabolomics study that reflect the cellular state under the experimental conditions studied. In many occasions the experiments are performed according to an experimental design to make sure that sufficient variation is induced in the data. However, as metabolomics is a holistic approach, also a large number of metabolites are measured in which no variation is induced by the experimental design. The presence of such non-induced metabolites hampers traditional data analysis methods as PCA to estimate the true model of the induced variation. The greediness of PCA leads to a clear overfit of the metabolomics data and can lead to a bad selection of important metabolites. Chapter 4 explores how, why and how severe PCA overfits data with an underlying experimental design. The results are compared to analysis of the same data with ASCA, a method that uses the prior knowledge of the experimental design as mentioned in section 1.4.2, to show the improvement of model estimation and reduction of overfit.

Longitudinal data plays an important role in the various fields of functional genomics to improve understanding and knowledge of the dynamics within biological systems. The time-resolved data of microbial metabolomics studies are expected to contain underlying dynamic profiles that are smooth. However, estimating these underlying smooth dynamic phenomena from such data is complicated due to the high complexity of the data and the limited number of techniques that can deal with this type of data. Traditional multivariate data analytical techniques, such as Principal Component Analysis, ignore the underlying dynamics in the data and give solutions that tend towards explaining variance rather than explaining dynamics and understanding biology. A new method is presented in Chapter 5 that uses the prior knowledge of expected smoothness in the underlying dynamics of the data by incorporating it into the data analysis. The method has been baptised with the name Weighted Smooth Principal Component Analysis (WSPCA) and incorporates smoothness into the scores of PCA by using a roughness penalty. For determination of the model meta parameters a cross-validation procedure is introduced based on predictions of elements randomly left out of the data. The method is applied to simulated noisy data containing true underlying smooth dynamic profiles to show the capability of capturing these profiles. Since the cross-validation is based on predictions of left out elements the method has also been applied to estimate truly missing data in a microbial metabolomics data set.

Chapter 6, the final one, contains a conclusion about the work described and an outlook for future research opportunities with respect to the use of biological knowledge in a microbial metabolomics study.

The chapters in this thesis represent a collection of articles, which are either published in or submitted to several scientific journals. As outlined in this section the chapters are linked into a framework but can and may be read independently of each other.