Unraveling substrate dynamics and identifying inhibitors in hydrolysates of lignocellulosic biomass by exometabolomics

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SUMMARY

Lignocellulosic biomass is the 2nd generation feedstock for biofuel production through fermentation processes. The resources are mostly agricultural and industrial wastes, which are inexpensive, environmental friendly and not competitive with world food-supply. Lignocellulosic biomass has a rigid structure, which is mainly composed of cellulose, hemicellulose and lignin. To break down the structure and expose cellulose and hemicellulose for hydrolysis, a pretreatment procedure needs to be applied. There are various ways to pretreat biomass, which differ in their severity and efficiency. A mild pretreatment method results in incomplete releasing of cellulose, while a harsh method may lead to the releasing and forming of toxic compounds through sugar and lignin degradation. These toxic compounds inhibit the growth of the fermenting micro-organism(s), which results in reduced productivity.

To improve the fermentability of the hydrolysis products of pretreated lignocellulosic biomass, the so called biomass hydrolysates, identifying their inhibitory compounds is of great importance. **Chapter 1** of this thesis reviews the approaches and techniques that have been used to study the inhibitors in various biomass hydrolysates, and introduces a non-targeted methodology to systematically identify biomass hydrolysate inhibitors: the exometabolomics approach. The four steps involved in this approach are: (i) defining research question, (ii) experimental design, (iii) sample selection and analysis and (iv) data analysis and interpretation.

To identify hydrolysate inhibitors through an exometabolomics approach, a wide range of biomass hydrolysates need to be prepared. Four pretreatment methods were developed and applied to six different biomass types, respectively. The fermentability of the resulting biomass hydrolysates were tested with the fermenting yeast, *Saccharomyces cerevisiae*. The detailed procedures of these four pretreatment methods are described in **Chapter 2**, which also reports the difference in fermentability among the generated biomass hydrolysates.

The hydrolysis efficiency of the pretreated biomass is further discussed in **Chapter 3**. To study hydrolysis beyond the formation of monosaccharides and understand the main oligosaccharide forms in biomass hydrolysates, high-performance anion-exchange chromatography coupled with mass spectrometry (HPAEC-MS) was applied. The method is able to separate and detect many oligosaccharides in one experimental run, and by using estimated response factors, the relative quantities of the detected oligosaccharides were

assessed. The analysis results revealed that, besides the monomers, disaccharides were the main remaining sugar form in all biomass hydrolysates generated by the pretreatment methods described in Chapter 2.

Chapters 4 to 6 are focused on identifying inhibitory compounds in lignocellulosic biomass hydrolysates and studying their effects on fermenting yeasts during fermentation processes. During biomass pretreatment processes hydrolysate inhibitors are released or formed, amongst which are mainly weak acids, furans and phenolic compounds (examples are acetic acid, furfural and vanillin, respectively). The presence and concentration of the inhibitors are biomass type and pretreatment method dependent, and inhibition effects include longer lag-phase, lower growth rate, and reduced productivity.

Chapter 4 reports the examination of the presence and dynamics of a target group of inhibitors in several different biomass hydrolysates. The fermentability of the hydrolysates were tested by conducting batch fermentations using baker's yeast, during which time-series samples were taken for their non-sugar composition analysis. The actual concentrations of the pre-selected inhibitors were determined, which are valuable references for toxicity tests of relevant potential inhibitory compounds.

Chapter 5 reports the detailed experimental procedure and results, in terms of the exometabolomics approach introduced in Chapter 1. The research question, identifying inhibitors in biomass hydrolysates, was answered by statistically correlating the fermentability of 16 different biomass hydrolysates with their non-sugar compositions. The composition analysis was conducted with two GC-MS methods to reach a high compound coverage, and the data analysis was realized by building statistical models. A list of compounds was identified as inhibitors in biomass hydrolysates, including novel compounds. The results suggest that metabolomics is a relevant approach in target identification in complex systems like lignocellulosic biomass hydrolysates.

Finally, in **Chapter 6**, we describe the isolation of microorganisms, from different environmental sources, that exhibit high resistance to biomass hydrolysate inhibitors. A unique strain was isolated and identified as *Pichia anomala*. This yeast showed relative high resistance in the tested hydrolysates compared to some other *P. anomala* strains, and was capable of utilizing xylose as C-source and nitrate as N-source. Through further research and possibly genetic modifications, this strain has the potential to become a suitable yeast for fermenting lignocellulosic biomass hydrolysates.