Enzymatic cascade reactions involving phosphorylated intermediates: immobilization and process optimization
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The research presented in this thesis describes the development of multi-enzymatic cascade reactions for the synthesis of natural and unnatural carbohydrates. The thermodynamic control of these cascades is based on the formation and hydrolysis of phosphorylated intermediates and the synthetic key step is the C-C bond formation. The enzymes used are acid phosphatase and DHAP-dependent aldolases. Possibilities of and limitations on the use of alkaline phosphatase and transketolase are also investigated. Another important part of this research involves the optimization of the efficiency of these cascade reactions through the development of more convenient processes. The immobilization of the enzymes to allow their reuse was the starting point to setup packed-bed reactors that allow the large-scale synthesis of valuable compounds with a continuous flow system.

The introductory Chapter 1 is divided in four parts covering the basic knowledge on the topics experimentally developed in the other chapters. The first section gives a general overview on the chemical and enzymatic methods used for the preparation of phosphomonoesters, with main focus on the use of acid and alkaline phosphatase. The second part deals with the enzymatic C-C bond formation. The different classes of aldolases are described with particular attention to DHAP-dependent aldolases. The third section explains the different strategies used in enzyme immobilization and highlights their pro and contra. The fourth and last part shortly describes the enzyme process technology with focus on the importance of the efficiency of processes for industrial biocatalytic application.

Chapter 2 describes a simple procedure for the synthesis of enantio- and diastereomerically pure carbohydrate analogues in one pot using a four-enzyme cascade reaction. Cheap and achiral precursors such as glycerol and aldehydes are transformed into multi-chiral molecules in a thermodynamically optimized sequence of reactions (phosphorylation – oxidation - aldol coupling - dephosphorylation), which lead to the complete conversion of the substrates. With this method the naturally occurring azasugar D-fagomine, a glycosidase inhibitor, was synthesized with 69 % yield. It was possible to obtain the other stereoisomers of the precursor of D-fagomine using the set of four DHAP-dependent aldolases. This method, which is very flexible with regard to the choice of aldehydes, allows the synthesis of different products and the
production of specific chiral configurations by using different aldolases. 
In Chapter 3 a protocol for the efficient immobilization of acid phosphatase from S. flexneri (PhoN-Sf) on epoxy-functionalized beads is described. A method for the large-scale synthesis of phosphorylated compounds is also developed. A continuous-flow packed-bed reactor with immobilized PhoN-Sf was used to produce D-glucose-6-phosphate, N-acetyl-D-glucosamine-6-phosphate, allylphosphate, glycerol-1-phosphate, inosine-5’-monophosphate, and N-acetyl-D-galactosamine-6-phosphate, dihydroxyacetone phosphate, and glyceraldehyde-3-phosphate from the corresponding primary alcohol using cheap pyrophosphate (PP_i) as phosphate donor. Most of these compounds were isolated on a gram scale. Some insights on the use of tripolyphosphate (PPP_i) reveal its usefulness in increasing the yields and reducing the amount of free phosphate produced.

Given the success of the research described in chapter 3, a three-steps cascade reaction with two immobilized enzymes in a flow reactor system is described in Chapter 4. The aldolases RAMA and RhuA were immobilized on beads with a good efficiency and used in the flow system in which the feed was dihydroxyacetone and an aldehyde. The sequence of reactions (phosphorylation - aldol coupling - dephosphorylation) was carried out with three sequential packed-bed columns. The precursor of D-fagomine and some isomers were obtained with this method.

In Chapter 5, the C 2-ketol elongation of aldehydes and aldose sugars by transketolase was investigated. The C-C coupling by transketolase was carried out with hydroxypyruvate as a substrate and using some of the phosphorylated aldose sugars obtained with the method described in Chapter 2. This yielded phosphorylated elongated ketose sugars. Transketolase was also immobilized with high efficiency on epoxy-functionalized beads and used together with PhoN-Sf in a complicated packed-bed flow reactor with two columns and three pumps to phosphorylate N-acetyl-D-glucosamine, which was then supposed to be elongated to produce a phosphorylated multi-chiral acetylated octulose. Unfortunately, this setup was not appropriate for the transketolase reaction but an alternative method is proposed.

In Chapter 6 the use of alkaline phosphatase as a substitute of PhoN-Sf in cascade reactions with aldolase is described. In contrast to acid phosphatase, alkaline phosphatase and aldolase are both active in the same pH range. This may result in higher yields when both enzymes are used in one pot. Unfortunately, the product phosphate, which is produced during the reaction, inhibited the soluble enzyme. However, the enzyme could phosphorylate dihydroxyacetone and glycerol to the same extent as PhoN-Sf. By immobilization of the alkaline phosphatase the phosphate inhibition could be
prevented, and good yields were obtained in the cascade reaction. Although alkaline phosphatase could not compete with acid phosphatase in our processes, it could still be a good alternative for the phosphorylation of compounds that are stable only under alkaline conditions.