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Investigation of the transglycosylation potential of ß-Galactosidase from *Aspergillus oryzae* in the presence of the ionic liquid [Bmim][PF_6]

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ABSTRACT

Finding efficient and simple methods for the synthesis of oligosaccharides is a relevant question for both pharmaceutical and food industry. An interesting route is transglycosylation by glycosidases due to their availability and cheap substrates. In this work transglycosylation by means of ß-galactosidase from *Aspergillus oryzae* was investigated at pH 4.5 using ONPG as glycosyl donor and several saccharides in different concentrations as acceptor. As the influence of ionic liquids was often described to be beneficial for synthesis or specificity of glycosidases, the ionic liquid [Bmim][Pf_6] was investigated in an emulsion with aqueous buffer. Additionally, a fed-batch mode is presented here, including the ionic liquid as extraction phase. Reaction products were analyzed via HPLC and thin-layer chromatography.

By use of transglycosylation, oligosaccharides were produced in high yields, often above 90%, both in aqueous buffer and the system using buffer and ionic liquid. The application of [Bmim][Pf_6] lead to an altered substrate specificity of ß-galactosidase: Depending on the system applied, preferentially di- or trisaccharides were produced, partly in contrast to the synthesis in aqueous buffer. Using a new model for the transglycosylation with ONPG reaction the observed reaction courses were well described.

The fed-batch mode, applying ONPG above solubility and using [Bmim][Pf_6] as extraction phase for ONP, strongly increased and product concentration. In this context it was observed that the absorption spectrum of ONP in [Bmim][Pf_6] is altered in the presence of an aqueous phase, even though [Bmim][Pf_6] is not mixable with water.

The findings in this work enable a better understanding of the influence of ionic liquids on enzyme activity and can help to make glycosidases an even more interesting tool in saccharide synthesis.

1. Introduction

The synthesis of oligosaccharides has been a subject of research for many years. This is due to the important role of saccharides in biological systems, e.g. protein glycosylation influences cell recognition, folding, solubility and charge of a protein [1,2]. Besides this, oligosaccharides are demanded by food and health supplement industries as additives for infant nutrition and edibles. As food additives, oligosaccharides can even be advantageous to health [3].

Still, efficient synthesis of higher oligosaccharides remains challenging. Many approaches have been applied, including chemical and enzymatic strategies. Chemical methods incorporate, for example, solid-phase and one-pot approaches [4,5], which improved applicability of chemical methods. However, they remain complex and time-consuming as many protection and deprotection reactions are necessary [6]. Therefore, enzymatic methods are an attractive alternative. Enzymes processing saccharides consist of two classes: transferases (EC 2) and hydrolases (EC 3) which are represented by glycosyltransferases, glycosidases, glycan phosphorylases and polysaccharide lyases. Glycosyltransferases, also called Leloir-transferases, are responsible for glycoside bond formation in cells. They catalyze the transfer of a monosaccharide unit from an activated sugar donor with phosphate leaving group (e.g. uridine diphosphate galactose, UDP-Galactose) to an acceptor and are highly specific both for sugar donor and acceptor [7]. The so-called non-Leloir Glycosyltransferases utilize activated substrates like sugar-phosphate and are related to phosphorylases. Despite their high specificity and efficiency, the use of glycosyltransferases is limited by the high costs of nucleotide sugars and their commercial availability [8].

Glycosidases are enzymes, which under natural conditions catalyze the hydrolysis of glycosidic linkages. Under appropriate conditions many glycosidases are capable of catalyzing the

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synthesis of glycosidic linkages [9]. Compared to glycosyltransferases, glycosidases are less regio- and stereoselective. As a consequence, different saccharides can serve as acceptors [8]. At the same time, lower selectivity may lead to product mixtures and the need for complex purification. As glycosidases are hydrolyzing enzymes, synthesis yields in water are usually low. Nevertheless, advantages are convincing: They are easily available, needless of cofactors and utilize simple substrates [8].

Synthesis can either follow equilibrium controlled or kinetically controlled conditions. For equilibrium controlled synthesis, also called reverse hydrolysis, glycosyl moieties are coupled by reversing the hydrolysis reaction. The hydrolysis reaction is as follows [8].

$$Gal - R + H_2O \leftrightharpoons Gal - H + R - OH$$

Whether glycosidic bonds are synthesized or cleaved only depends on the equilibrium of the reaction, which is influenced by factors like substrate concentration and water activity. Thus, synthesis can be achieved by increasing reactant concentration, decreasing water activity or removing product from the reaction solution. However, synthesis yields are usually low in this system [8],

The hydrolysis reaction catalyzed by glycosidases is a transfer reaction with an enzyme-substrate intermediate. During hydrolysis the glycosyl moiety of the substrate is transferred to water. With an appropriate alternative offered, the glycosyl moiety can be transferred to an acceptor molecule usually with a hydroxyl group – like a saccharide [8].

For kinetically controlled synthesis, an activated glycosyl donor like lactose or ONPG is used [10]. After being bound by the enzyme, the glycosyl moiety in the enzyme-substrate intermediate will be trapped by the glycosyl acceptor faster than by water (see Fig. 1). With high donor and acceptor concentration present, synthesis of glycosidic bonds is favored kinetically, whereas hydrolysis of donor and product is favored thermodynamically. Due to this competition a maximum for product concentration will be reached [11]. Competing reactions in this system are the formation of trisaccharides, resulting from the condensation of ONPG and a formerly produced disaccharide. Both disaccharides and trisaccharides are also subject to hydrolysis (secondary hydrolysis). Yields are generally higher with transglycosylation than with reverse hydrolysis. Still, high concentrations of saccharide donor and acceptor are necessary.

In this study, ß-galactosidase from *Aspergillus oryzae* (*A. oryzae*) was applied for transglycosylation reactions. ß-Galactosidase is a highly studied enzyme and well available due to its industrial relevance. In food industry ß-galactosidase is applied for the cleavage of lactose e.g. in milk products. Additionally, it could be used for synthesis of galacto-oligosaccharides (GOSs), which have potential beneficial probiotic effects [12].

The long known and applied synthesis of saccharides by means of ß-galactosidase is examined in the presence of the ionic liquid (IL) 1-Butyl-3-methylimidazolium hexafluorophosphate ([Bmim][PF_6]) in this work. The use of ionic liquids in the synthesis of saccharides via glycosidases goes back to the early 2000s, when Kaftzik et al. [13] introduced the production of *N*-acetyllactosamine from lactose and *N*-acetylglucosamine by use of ß-galactosidase from *Bacillus circulans* (*B. circulans*). The water soluble ionic liquid 1,3-di-methyl-imidazol-methyl sulfate [MMIM] [$MeSO_4$] lead to a doubled yield when used in a ratio of 25%. The authors state that the reduced water activity might be the cause for the observed decreased secondary hydrolysis. However, the effect of the IL seems to be larger than expected, taking into account the strong increase of yield in relation to the reduction of water activity. These observations showed that the effect of IL on the synthesis of saccharides via glycosidases is hardly predictable.

Since then, several groups investigated the effect of different IL on transglycosylation, usually aiming at specific products like arylalkyl glycopyranosides [14] or *N*-acetyl-galactosides [15,16]. Often altered yields and stereospecificities were the results. In 2012 Sandoval et al. investigated possible causes for the observed changes by means of SPR and circular dichroism [16]. However, a comparative study regarding different saccharide acceptors and concentrations with and without the presence of IL is still missing, although such a study could be of high relevance for the production of nutritional saccharides. In this work, we investigate the yields for transglycosylation reactions with ONPG as glycosyl donor and the acceptors mannose, glucose and galactose in different concentrations. Thereby, both disaccharide and trisaccharide products were explored and, for the first time, the extraction of ONP in the IL was monitored. Additionally, a model for the transglycosylation reaction is presented which incorporates the hydrolysis of ONPG as glycosyl donor and a specific saccharide as glycosyl acceptor. This is in contrast to common models, in which lactose serves as glycosyl donor [17–20]. The use of ONPG as glycosyl donor offers several advantages. First, it allows for a simple photometric analysis of the reaction product ONP. And Second, ONP does not take part in further transglycosylation reactions, other than the hydrolysis products of lactose, thus keeping the reaction network relatively simple and allowing for more reliable quantitative models.

2. Material and methods

O-Nitrophenyl-β-D-galactopyranoside (ONPG), β-galactosidase (EC 3.2.1.23) from *A. oryzae,* D-(+)-galactose, D-(+)-glucose, D-lactose monohydrate, D-(+)-melezitose monohydrate, D-(+) raffinose pentahydrate, Acetonitrile (HPLC grade) and 2-nitrophenol (ONP) were purchased from Sigma Aldrich (St. Louis, Missouri, USA). D-(+) mannose, maltotriose and the ionic liquid 1-*n*-butyl-3-methylimidazolium (98+%) ([Bmim][PF_6]) were purchased from Alfa Aesar (Ward Hill, Massachusetts, USA). Isopropanol and ethyl acetate, both of analytical grades, were purchased from VWR (Radnor, Pennsylvania, USA).

2.1. Buffers

The synthesis of oligosaccharides was performed in citrate phosphate buffer at pH 4.5. The buffer was prepared according to McIlvaine [21].

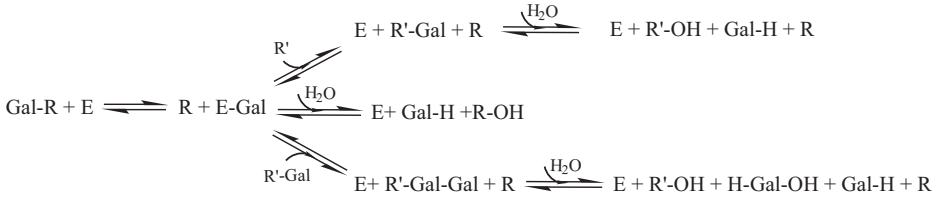

Fig. 1. Transglycosylation reaction scheme for ß-galactosidase.