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Use of Ionic Liquids to Increase the Yield and Enzyme Stability in the β-Galactosidase Catalysed Synthesis of *N*-Acetyllactosamine

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Abstract:

The use of ionic liquids as alternative solvents for enzyme catalysis was investigated. β-Galactosidase from *Bacillus circulans* catalyses the synthesis of *N*-acetyllactosamine starting from lactose and *N*-acetylglucosamine in a transglycoslyation reaction. The addition of 25% v/v of 1,3-di-methyl-imidazol-methyl sulfate [MMIM] [$MeSO_4$] as a water-miscible ionic liquid suppresses the secondary hydrolysis of the formed product, resulting in doubling the yield to almost 60%. The enzyme can be reused several times after ultrafiltration of the reaction mixture without loss of activity. Results of different amounts of ionic liquids in the reaction medium on the thermostability of the galactosidase as well as on oxidoreductases are presented as well.

Introduction

During the past decade ionic liquids have gained increasing attention for performing all types of reaction with sometimes remarkable results.[1,2] Ionic liquids are low-melting (<100 °C) salts which represent a new class of nonmolecular, ionic solvents. They have many properties which make them of fundamental interest to all chemists, since both the thermodynamics and kinetics of reactions carried out in ionic liquids are different to those in conventional molecular solvents. Therefore, there are many good reasons to study ionic liquids as alternative solvents in enzyme catalysis as well.

Besides the engineering and environmental advantage[3] of their nonvolatile nature, the investigation of new biphasic reactions is of special interest. In transition metal-catalyzed reactions, for example, it has been found in many cases that ionic liquids can act as a superior solvent in comparison to water and common organic solvents, especially when ionic complexes are used as catalysts.[4–6] In these cases, significant enhancement of catalyst activity and stability has been possible. Catalysts for enantioselective reactions have been recycled using ionic liquids as well.[7] Due to their special properties and possible advantages ionic liquids may be ideal solvents for biocatalytic reactions as well so that some problems such as substrate solubility, yield, enzyme stability or selectivity may be solved.

Generally, there are three modes to operate ionic liquids in a biocatalytic process: (i) use as a pure solvent, (ii) use as a cosolvent in aqueous systems, or (iii) as a cosolvent in a biphasic system. After trials first using ethylammonium nitrate in mixtures with water more than 15 years ago,[8] in the last two years a number of papers were published on first results of the use of pure ionic liquids as reaction medium for enzymatic reactions. In these examples ionic liquids such as [BMIM] [PF_6] or [BMIM] [BF_4] have been used to replace organic solvents in protease- and lipase-catalysed reactions.[9–15] A biphasic system containing an ionic liquid for in situ product extraction for a whole-cell process[16] and also the use of simple ionic liquids for protein renaturation have been described as well.[17]

Results and Discussion

Enzyme Activity in the Presence of Ionic Liquids. The influence of different ionic liquids[18] on the activity of hydrolases (β-galactosidase from *Bacillus circulans*, lipases from different origins) and oxidoreductases (formate dehydrogenase (FDH) from *Candida boidinii*, alcohol dehydrogenase (YADH) from *yeast*, carbonylreductase (CPCR) from *Candida parapsilosis*) was investigated. The results of the lipase-catalysed resolution of phenylethanol in pure ionic liquids with improved enantioselectivity compared to methyl-*tert*-butyl ether (MTBE) as reference solvent have been published elsewhere.[19] For the other enzymes the activity in dependence on the content of ionic liquid in the aqueous buffer solution were studied using established photometric enzyme assays. The results obtained for the FDH assay and the β-galactosidase assay are summarized in Table 1.

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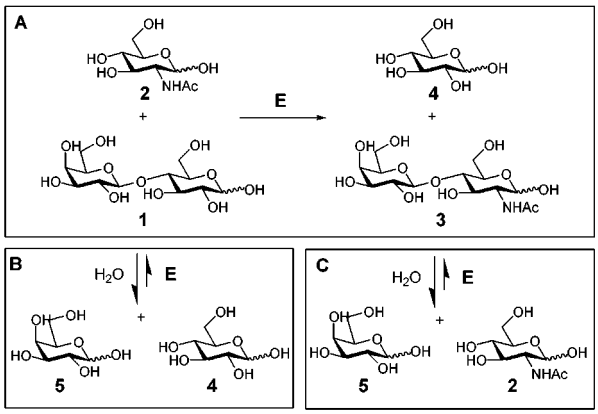
Table 1. **Enzyme activity in the presence of ionic liquid, % of residual activity in comparison to the activity in pure buffer solution**

	FDH			β-galactosidase		
Ionic liquid [% v/v]	25	50	75	25	50	75
[MMIM] [$MeSO_4$]	65	73	98	74	14	–
[Et_3NH] [$MeSO_4$]	–	–	–	–	–	–
[Et_3NMe] [$MeSO_4$]	82	55	–	–	–	–
[$PrNH_3$] [NO_3]	–	–	–	–	–	–
[BMIM] [BF_4]	–	–	–	31	–	6
[EMIM] [$PhCO_2$]	–	–	–	–	–	–
[BMIM] [F_3CSO_3]	38	3	–	–	–	–
[BMIM] [$OctSO_4$]	–	–	–	35	10	–

The best results were obtained for FDH and β-galactosidase in mixtures of the ionic liquid 1,3-dimethyl-imidazolmethyl sulfate [MMIM][$MeSO_4$] with buffer. The YADH-catalysed oxidation of ethanol was only possible in the presence of 25% v/v of [BMIM][F_3CSO_3] with a residual activity of less than 5%. However, it must be pointed out that possible impurities in the ionic liquids which are difficult to analyse and to remove may be the reason for the loss of enzyme activity. For the CPCR-catalysed reduction acetophenone was used as substrate. The low solubility of acetophenone in aqueous media can be increased 2- to 3-fold in the presence of 25% (v/v) [MMIM] [MeSO4]. However, thus far no activity could be found for CPCR in the presence of the ionic liquids investigated. This is surprising because for this enzyme a good tolerance against organic solvents has been reported.[20,21] For FDH which can be used for cofactor regeneration in the CPCR-catalysed reaction even higher contents of ionic liquids were possible without reduction of the enzyme's activity.

β-Galactosidase-Catalyzed Synthesis of *N*-Acetyllactosamine. In the following we focused our work on the behaviour of β-galactosidase in the presence of ionic liquids. The use of enzymes for the synthesis of di- and oligosaccharides has been established more than a decade ago.[22−24] The commercially available β-galactosidase from *B. circulans*[25] shows a remarkable β-1,4 selectivity and oligosaccharide synthesis results in high regioisomeric purity of the synthesized product.[26,27] Oligosaccharides such as *N*-acetyllactosamine (LacNAc) have attracted much interest in immunological and pharmacological research because of their significance as fundamental structures of glycoproteins and glycolipids.[28−30] D-Galacto-oligosaccharides have been synthesized mainly by utilizing the transglycosylation activity of β-galactosidase of various origins.[24,27,31−33]

Scheme 1. **Enzyme-catalyzed synthesis of *N*-acetyllactosamine 3; the enzyme E is β-galactosidase (A) transgalactosylation, (B) hydrolysis of 1 (side reaction), (C) hydrolysis of the product 3 (secondary hydrolysis)**



Scheme 1 shows the reaction scheme of the transglycosylation described here. Starting with lactose **1** transgalactosylation results in the transfer of the galactose moiety of **1** to *N*-acetylglucosamine **2** to afford *N*-acetyllactosamine **3** (A). The galactosyl donor **1** is hydrolysed to glucose **4** and galactose **5** in a side reaction (B). The desired product **3** is also a substrate for the β-galactosidase. When the reaction is performed in water, the yield is limited to 30% due to the secondary hydrolysis of **3** in a subsequent reaction by the enzyme (C). However, it is difficult to separate the product from the starting disaccharide and also to determine the optimum reaction time to terminate the reaction to obtain high yield of the desired product. As we have previously reported this can be advantageously realised in a continuously operated stirred tank reactor with retention of the enzyme by an ultrafiltration membrane.[34]

There have been studies to increase the yield of *N*-acetyllactosamine by addition of organic cosolvents such as 2-ethoxy ethyl ether and trimethyl phosphate, but starting with lactose as a cheap galactose donor with 2-ethoxy ethyl ether only a yield higher than 20% could be achieved. Higher yields of 30−40% are only possible with more expensive donors such as *p*-nitrophenylgalactoside as substrates.[35,36]

Another approach is the addition of high salt concentrations. Kren and co-workers reported the increase of the yield from 2 to 15% by addition of salt concentrations of up to 1 mol/L.[37]

Influence of Ionic Liquids on Yield and Selectivity of Disaccharide Synthesis. In the following the results of the synthesis of *N*-acetyllactosamine in the presence of ionic liquids are presented. To compare the results with those published earlier, 25 °C was chosen as reaction temperature. Accordingly, the concentrations were in the same order of

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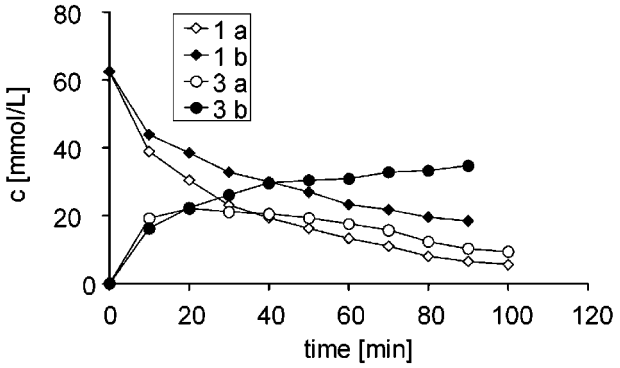

Figure 1. **Concentration as function of time for the synthesis of *N*-acetyllactosamine 3 in a batch reactor without (open symbols) and with ionic liquid (filled symbols); starting concentrations: (a) 62 mmol/L lactose 1, 600 mmol/L *N*-acetylglucosamine 2, 2 mg/mL β-galactosidase, 195 mmol/L K_2HPO_4, 65 mmol/L KH_2PO_4, pH 7.3, 23 °C; (b) 62 mmol/L lactose 1, 600 mmol/L *N*-acetylglucosamine 2, 2 mg/mL β-galactosidase, 195 mmol/L K_2HPO_4, 65 mmol/L KH_2PO_4, 25% (v/v) [MMIM] [$MeSO_4$], pH 7.3, 23 °C. The amount of 3 (●) and 1 (◆) produced/hydrolyzed as a function of time were examined, and samples were analyzed by HPLC.**

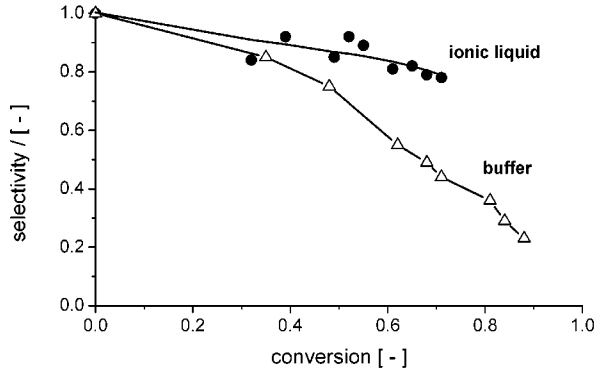

Figure 2. **Improved selectivity in ionic liquids for the enzymatic synthesis of *N*-acetyllactosamine (LacNAc) due to suppression of the secondary hydrolysis. Conditions: 62 mmol/L Lac, 600 mmol/L GlcNAc, 25% (v/v) [MMIM][$MeSO_4$], 2 g/L β-galactosidase, 25 °C, pH 7.3. Selectivity is the ratio of LacNAc 3 formed to lactose 1 consumed. The product (conversion × selectivity) is the yield of the reaction.**

magnitude. Table 1 shows a decrease of β-galactosidase activity with increasing ionic liquid content. Therefore, its amount was limited to 25% (v/v). Under these conditions the maximum activity was obtained in the presence of 25% (v/v) [MMIM][$MeSO_4$]. In Figure 1 a reaction in buffer (open symbols) is compared to that with the addition of ionic liquid (solid symbols). In buffer the concentration of *N*-acetyllactosamine goes through a maximum which corresponds to a yield of 30% caused by the secondary hydrolysis. In the presence of [MMIM][$MeSO_4$] the secondary hydrolysis is suppressed, resulting in final product concentrations of 36 mmol/L corresponding to a yield of 58%. As can be seen from Figure 1 the reaction velocity of product formation is not influenced by the presence of the ionic liquid. In Figure 2 the selectivity is shown as a function of conversion. The enzyme shows a better selectivity in ionic liquid over a long time. There is no need to determine the optimum reaction time to terminate the reaction to obtain high yields of the

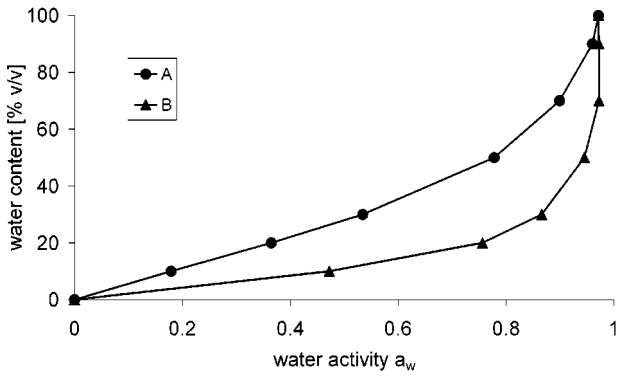

Figure 3. **Water activity (a_w) as function of water content [% (v/v)] in different water−ionic liquid mixtures at 25 °C; (A) [MMIM] [$MeSO_4$], (B) [BMIM] [$OctSO_4$].**

transgalactosidation product. The final product/educt ratio of disaccharide of 2:1 obtained is another advantage facilitating product isolation.

To present a real alternative to synthesis in water or organic solvents, it is important to demonstrate that ionic liquids can be recycled after the reaction and product separation is possible. If the ionic liquid used in this experiment is not miscible with organic solvents, the product isolation is possible via simple extraction. However, in this case that cannot be realized, because of the low solubility of the saccharides in organic solvents. Product isolation is possible by using chromatography methods as published earlier.[38] The product isolated within this method still contains small amounts of the ionic liquid, but modern nanofiltration membranes allow the separation of the noncharged product from the ionic liquid.[39,40] Because of the high cost of enzymes it is also important to separate the enzyme so that it can be reused several times. In addition to the immobilisation[41] of enzymes, ultrafiltration is a good method to separate a soluble enzyme from the reaction mixture.[34,42] The β-galactosidase is stable under the conditions employed, allowing its repeated use after filtration with an ultrafiltration membrane. In this way, enzyme stability can be investigated in the presence of ionic liquid under reaction conditions. After three times of repeated use in the presence of [MMIM] [$MeSO_4$], no reduction of activity was observed.[43]

The somewhat lower reaction rate of the hydrolysis of both lactose and *N*-acetyllactosamine could be correlated with a reduction of water activity (a_w) caused by the ionic liquid. In the presence of 25% (v/v) [MMIM] [$MeSO_4$] for a_w a value of 0.83 was obtained. As shown in Figure 3, the water activity in the presence of 25% (v/v) [MMIM] [$MeSO_4$] is lower than in the presence of 25% (v/v) [BMIM] [$OctSO_4$].[44]

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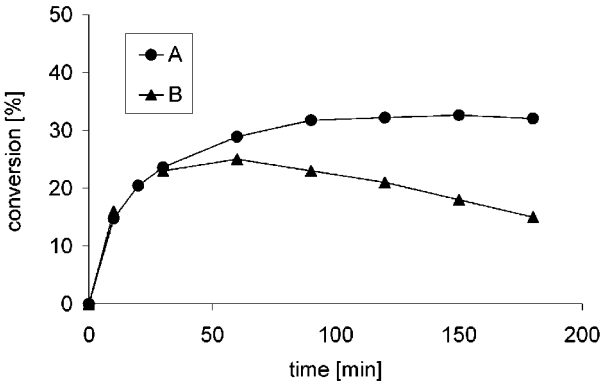

Figure 4. **Conversion as function of time for the synthesis of *N*-acetyllactosamine in the presence of different ionic liquids resulting in different water activities; starting concentrations: 62 mmol/L lactose, 600 mmol/L *N*-acetylglucosamine, 2 mg/mL β-galactosidase, 195 mmol/L K_2HPO_4, 65 mmol/L KH_2PO_4, pH 7.3; 23 °C. (A) 20% (v/v) [MMIM] [$MeSO_4$]. (B) 20% (v/v) [BMIM] [$OctSO_4$].**

This might be due to the stronger interaction between the methyl sulfate ions and the water molecules. The lower water activity may be the reason for suppression of the secondary hydrolysis as shown in Figure 4. However, the effects observed may be also ascribed to interactions of the components of the ionic liquid with charged groups in or near the active site of the enzyme. Studies about the reasons for the improved yield revealed that simple salts such as sodium chloride can be used in a similar way. A saturated solution of sodium chloride has an a_w value of 0.75 and a 25% (w/w) solution of 0.88, but the use of sodium chloride is limited by its solubility in the reaction media, while [MMIM][$MeSO_4$] is totally miscible with water without limitations. Another important advantage of the ionic liquid as cosolvent is the increase in the solubility of hydrophobic components.

Solvent Effect on Enzyme Stability. It has been shown that the stability of enzymes is markedly increased under low water conditions,[45] making it possible to perform biotransformations at temperatures higher than those used in conventional aqueous solutions. The most common cause for the inactivation of enzymes at elevated temperatures is the loss of the native, catalytically active conformation. Recently the work of Turner et al.[46] demonstrated that the temperature at which a protein undergoes thermal denaturation (T_d) is strongly dependent on the amount of water associated with the protein.[47] It was found that the thermal denaturation could be correlated with the water activity (a_w) of the sample.[48−50] With lowering the water activity of the medium T_d increases. After 60 h of incubation in different ionic liquids (100% (v/v)), water, and saturated sodium chloride solution at 50 °C, the β-galactosidase remained active only in [MMIM][$MeSO_4$] and water (Figure 5). A rapid enzyme inactivation is observed in a predominantly

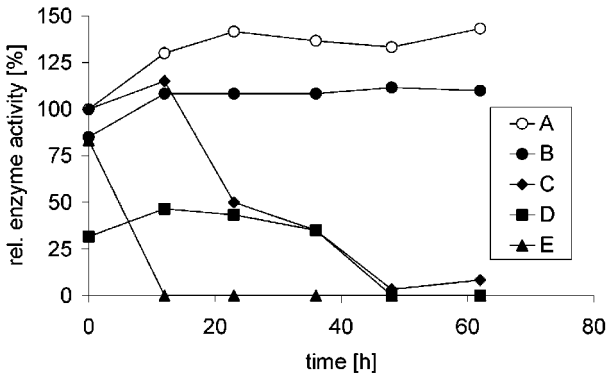

Figure 5. **Time courses of β-galactosidase inactivation at 50 °C in: (A) 100% (v/v) H_2O, (B) 100% (v/v) [MMIM] [$MeSO_4$], (C) 100% (v/v) [BMIM] [$MeSO_4$], (D) 100% (v/v) [$MNEt_3$] [$MeSO_4$], (E) 100% (v/v) saturated NaCl solution. The enzymatic stability was determined by measuring the residual hydrolytic activity with *o*-nitrophenylgalactoside in water as described in the Experimental Section. The initial activity was measured at 23 °C and was set to 100% for water as standard.**

aqueous medium at 80 °C, whereas in 100% (v/v) [MMIM]-[$MeSO_4$] the enzyme retained 71% of its initial hydrolytic activity after 12 h of incubation. Unfortunately, these conditions do not offer further advantages for the synthesis of *N*-acetyllactosamine besides an increased enzyme stability. At higher ionic liquid content the enzyme activity strongly decreases. This might be due to mass-transport limitations due to the higher viscosity of the reaction medium, even at higher temperatures.

Conclusions and Outlook

These results clearly demonstrate that ionic liquids can be used advantageously in enzymatic transglycosylation reactions. Not only can they replace organic solvents in these reactions, but they can also offer the advantage of altering the reactions conditions in an advantageous way. From initial investigation it becomes obvious that ionic liquids offer a useful method to control the water activity in water-rich environments in a different manner from simple salts. It should be noted that the ionic liquids are probably not fully dissociated when placed in an aqueous environment. Thus, the conductivity of these solutions is quite low. Ion pairs and larger aggregates may also be the reason for the improved solubility of hydrophobic compounds in mixtures of water and ionic liquids. The influence of water activity in nonmiscible ionic liquids has been demonstrated recently.[51,52]

On the other hand it is very likely that there are interactions between the compounds of an ionic liquid and charged groups of the enzyme as well. The interaction might be the reason for changes in the enzyme's selectivity and stability. Here the effects observed resemble to the of the ectoines, a class of zwitterionic compounds found to protect and stabilise proteins.[53]

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Experimental Section

Materials. β-Galactosidase was purchased from Daiwa Kasei, Osaka, Japan. All other chemicals were from Fluka, Buchs, Switzerland. The ionic liquids were kindly donated by the group of Dr. Wasserscheid, RWTH Aachen. CPCR and FDH was a gift from Professor Kula, Heinrich-Heine-University, Düsseldorf.

Enzyme Assay Formate Dehydrogenase. The reduction of β-nicotinamide adenine dinucleotide (NAD^+) with simultaneous oxidation of formate was used. Aqueous sodium formate solution (0.1 mL, 2.4 mol/L) and enzyme solution (0.1 mL, FDH 0.7 mg/mL, 8.4 U, containing 6 mmol/L NAD) were added to 1 mL of buffer (50 mM triethanolamine hydrochloride, 1 mmol/L dithiothreitole, HCl, pH 7). The increase of NADH was measured at 25 °C using a spectrophotometer at 340 nm. For the reactions in the presence of different % v/v of ionic liquid the amount of buffer was reduced and replaced by the ionic liquid.

Enzyme Assay β-Galactosidase. Hydrolysis of *o*-nitrophenylgalactoside to *o*-nitrophenol und galactose was used. Aqueous *o*-nitrophenylgalactoside solution (0.1 mL, 30 mmol/L) and aqueous enzyme solution (0.1 mL of β-galactosidase 10 mg/mL) were added to 1 mL of buffer (65 mmol/L KH_2PO_4, 195 mmol/L K_2HPO_4, pH 7.3). The increase of *o*-nitrophenol was measured at 25 °C using a spectrophotometer at 405 nm. For the reactions in the presence of different % v/v of ionic liquid the amount of buffer was reduced and replaced by the ionic liquid.

Enzyme Assay Yeast Alcohol Dehydrogenase. The oxidation of ethanol with simultaneous reduction of β-nicotinamide adenine dinucleotide (NAD^+) was used. Aqueous ethanol solution (0.1 mL, 7.2 mol/L), NAD^+ solution (0.1 mL, 1.2 mmol/L), and enzyme solution (0.01 mL, YADH 30 U/mL) were added to 0.99 mL of buffer (65 mmol/L KH_2PO_4, 195 mmol/L K_2HPO_4, pH 7.3). The increase of NADH was measured at 25 °C using a spectrophotometer at 340 nm. For the reactions in the presence of different % v/v of ionic liquid the amount of buffer was reduced and replaced by the ionic liquid.

Enzyme Assay CPCR. Reduction of acetophenone under oxidation of β-nicotinamide adenine dinucleotide (NADH) was used. β-Nicotinamide adenine dinucleotide solution (0.1 mL, NADH 2 mmol/L in buffer) and enzyme solution (0.02 mL, CPCR 2−3 U/mL in glycerin) were added to 0.88 mL of acetophenone solution (10 mmol/L acetophenone, 100 mmol/L TEA/NaOH, pH 7.0). The decrease of NADH was measured at 37 °C using a spectrophotometer at 340 nm. For the reactions in the presence of different % v/v of ionic liquid the amount of buffer was reduced and replaced by the ionic liquid.

Measurement of Water Activity. The water activity (a_w) in different water/ionic liquid mixtures was measured with an a_w measuring instrument from Novasina (Novasina AW SPRINT, Axair Ltd. Switzerland).

Synthesis of *N*-Acetyllactosamine in a Batch Reactor. All reactions were carried out in 1.5-mL flasks at room temperature. Enzyme solution (0.2 mL, 10 mg/mL in buffer) was added to a mixture containing 0.05 mL of buffer (65 mmol/L KH_2PO_4, 195 mmol/L K_2HPO_4, pH 7.3), *N*-acetylglucosamine solution (0.5 mL, GlcNAc 1.2 mol/L in buffer), and lactose solution (0.25 mL, 250 mmol/L in buffer). For the reactions in the presence of ionic liquid 0.2 mL of enzyme solution (10 mg/mL in buffer) was added to a mixture containing 0.05 mL of buffer (65 mmol/L KH_2PO_4, 195 mmol/L K_2HPO_4, pH 7.3), 0.5 mL of *N*-acetylglucosamine solution (GlcNAc 1.2 mol/L in 2:1 buffer/ionic liquid), and 0.25 mL of lactose solution (250 mmol/L in 2:1 buffer/ionic liquid). The reactions were terminated by thermal denaturation of the enzyme (10 min at 100 °C). The samples were filtered (Minisart RC 4 from Sartorius) to remove proteins and analysed by HPLC using a Aminex HPX-87H column from BioRad, Munich, Germany, 0.006 mol/L sulfuric acid as eluent with a flow of 0.8 mL/min at 65 °C; detection was done using an UV detector at 208 nm and a RI detector.

Recovery and Repeated Use of β-Galactosidase by Ultrafiltration. Six milliliters of enzyme solution (10 mg/mL in buffer) was added to a substrate solution containing 1.5 mL of buffer (65 mmol/L KH_2PO_4, 195 mmol/L K_2HPO_4, pH 7.3), 15 mL of *N*-acetylglucosamine solution (GlcNAc 1.2 mol/L in 2:1 buffer/ionic liquid) and 7.5 mL of lactose solution (250 mmol/L in 2:1 buffer/ionic liquid) and mixed in an ultrafiltration cell (Schleicher and Schuell, V_{max}: 80 mL, P_{max}: 6 bar, equipped with a membrane YM 3, cutoff 3000 g/mol) for 10 min. The ultrafiltration cell was kept at 23 °C. By pressurizing the cell with argon the solution containing product and nonreacted substrates is separated from the enzyme. The retentate is concentrated to 6 mL. Twenty-four milliliters of fresh substrate solution is added to the remaining solution in the filtration cell and treated as the first batch. The filtrate is diluted with water and analyzed by HPLC. Part of the solution obtained was purified as desribed in the literature.[38,54] The resulting LacNAc (20 mg) was identical to a commercial sample. Impurities of ionic liquid still present did not interfere with the analytical methods.

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