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Enzymatic Production and Complete Nuclear Magnetic Resonance Assignment of the Sugar Lactulose

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The enzymatic transgalactosylation from lactose to fructose leading to the prebiotic disaccharide lactulose was investigated using the β-galactosidase from *Aspergillus oryzae* and the hyperthermostable β-glycosidase from *Pyrococcus furiosus* (CelB). The conditions for highest lactulose yields relative to the initial lactose concentration were established on a 1 mL scale. Dependent on the initial molar ratio of lactose to fructose, more or fewer oligosaccharides other than lactulose were generated. Bioconversions on a 30 mL scale in a stirred glass reactor were performed, and lactulose yields of 46 mmol/L (44% relative to lactose) for CelB and 30 mmol/L (30% relative to lactose) for *A. oryzae* β-galactosidase were achieved. Only <5% of other oligosaccharides were detectable. The corresponding productivities were 24 and 16 mmol/L/h, respectively. The molecular structure of lactulose was investigated in detail and confirmed after purification of the reaction solution by LC-MS and 1D and 2D NMR. Lactulose (4-*O*-β-D-galactopyranosyl-D-fructose) was unambiguously proved to be the major transglycosylation disaccharide.

KEYWORDS: Lactulose; lactose; fructose; β-galactosidase; CelB; *Pyrococcus furiosus*; *Aspergillus oryzae*; transgalactosylation; biotransformation; NMR; LC-NMR; MS

INTRODUCTION

As a result of worldwide cheese production the disaccharide lactose accumulates in whey to an amount of ∼4 million tons per year (*1*). Only 25% of this amount is used for further applications. The residual amount of 3 million tons per year causes increasing environmental problems by its disposal (*2*). Utilization in food and pharmaceuticals is very limited, because ∼70% of the world population is lactose intolerant, leading to serious gastrointestinal problems after lactose consumption (*3*). Therefore, it is of commercial interest to develop alternative methods of exploitation of the renewable raw material lactose.

One compound of industrial interest related to lactose is lactulose (4-*O*-β-D-galactopyranosyl-D-fructose). It is formed in small amounts by the isomerization of lactose during the preservation of milk, that is, pasteurization, sterilization, or ultrahigh heat treatment (*4*). Lactulose is a sugar with greater sweetness and better solubility than lactose, and it contributes the bifido-factor in nutrition. It is also known to be an important constitutional factor in infant formula products (*5*). Furthermore, lactulose has several applications in the food and pharmaceutical fields. It is of considerable medical interest for the treatment of portal systemic encephalophady and chronic constipation and is added to commercial infant formula products as well as various milk products (*6*, *7*). Additionally, lactulose can be used as a sweetener for diabetics, as a sugar substitute in confectionery products, as a type of yogurt additive in milk/dairy applications, and in various liquid or dried food preparations routinely manufactured for older humans (*7*, *8*). Recently, the term "prebiotic" was introduced in food science for "a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon" (*9*). Thus, lactulose can be designated a prebiotic in human health (*5*, *10*, *11*).

Lactulose can be chemically synthesized by alkaline isomerization of lactose. To obtain high yields, it is necessary to use high amounts of catalysts, such as boric acid or aluminate (*6*). The waste management and product purification of the chemical process are cost intensive. Unfortunately, up to now no enzyme has been described catalyzing the direct isomerization of lactose to lactulose, although an enzymatic production of lactulose would be advantageous in the context of waste management, product purification, and the valuable status of "natural product" in the food industry.

However, generally two enzyme classes, namely, glycosyltransferases and glycosidases, are known to catalyze the formation of disaccharides (*12*). From an industrial point of view the glycosyltransferases are less appropriate because they usually need activated substrates and are not available in sufficient

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amounts. Using the cofactor-independent glycosidases for disaccharide synthesis, two reaction routes can lead to condensation products. These are the so-called "reverse hydrolysis", starting with monosaccharides and being a thermodynamically controlled reaction, and the "transglycosylation", starting with a typical substrate such as lactose (galactose donor) in the case of β-galactosidases in the presence of a monosaccharide (galactose acceptor), which is a kinetically controlled process. The latter reaction strategy was chosen for enzymatic lactulose synthesis.

β-Galactosidases from different sources, including permeabilized cells of *Kluyveromyces lactis* and β-galactosidase from *Aspergillus oryzae*, are described as catalysts for the transgalactosylation from lactose to fructose resulting in lactulose (*13*, *14*). Recently, a hyperthermostable β-glycosidase (EC 3.2.1.21) from *Pyrococcus furiosus* (CelB) was successfully used for lactose hydrolysis and galacto-oligosaccharide formation, respectively (*15*−*21*). Thus, the β-glycosidase from *P. furiosus* was employed for biotransformations using lactose as glycosyl donor and fructose as acceptor to produce prebiotic galactosyl-fructoses such as lactulose. The β-glycosidase featured mainly β-glucosidase activity (100%) as well as β-galactosidase activity (60%), and it has a half-life of 85 h at 100 °C (*22*, *23*).

In this study, the efficiency of a hyperthermostable β-glycosidase from *P. furiosus* toward the formation of galactosyl-fructoses starting from lactose and fructose was investigated, and the results were compared with a commercially available β-galactosidase from *A. oryzae*. Furthermore, the resulting main galactosyl-fructose product was purified, and its molecular structure was estimated by NMR studies.

MATERIALS AND METHODS

Chemicals and Enzymes. All chemicals of analytical grade or higher were purchased from Sigma-Aldrich (Seelze, Germany). Eupergit C was a kind gift from Roehm GmbH (Darmstadt, Germany). β-Galactosidase from *A. oryzae* was purchased from Sigma-Aldrich (Seelze, Germany).

Purification and Immobilization of CelB. β-Glycosidase from *P. furiosus* (CelB) was produced heterologously in *Escherichia coli* BL21 (DE3) pLUW511 and partially purified after cell disruption by heat treatment and dialysis (*22*, *24*). The partially purified enzyme was lyophilized and used as free enzyme and for immobilization on Eupergit C.

The immobilization of CelB on Eupergit C was carried out according to a modified method of Fischer et al. (*22*). Dry Eupergit C (2.5 g) was washed with 500 mL of distilled water. Phosphate buffer (25 mL; 1 mol/L, pH 5.5, 0.8 mol/L lactose) and the partially purified enzyme (40 mg, lyophilized, 4400 nkat) were added. The suspension was shaken for 24 h at 50 °C in a 50 mL flask, then applied on a suction filter, and washed with 1000 mL of phosphate buffer (50 mmol/L, pH 5.5) followed by 200 mL of water, 500 mL of Tris buffer (50 mmol/L, pH 7.5), 1000 mL of water, and finally 200 mL of acetate buffer (50 mmol/L, pH 5.0) (*22*). The immobilized biocatalyst had an activity of 1.5 nkat/mg of dry biocatalyst. The immobilization yield was 82.5% of the initially applied activity. The immobilized CelB was air-dried at room temperature for 5 h and kept in the freezer at −18 °C.

Enzyme Activity Measurement. CelB and *A. oryzae* β-galactosidase activities were assayed in 1 mL scale using *p*-nitrophenyl-β-D-galactopyranoside as substrate at 75 and 37 °C, respectively. For the assays a final concentration of 25 mmol/L *p*-nitrophenyl-β-D-galactopyranoside dissolved in acetate buffer (50 mmol/L, pH 5.0) was used. Substrate and enzyme solution were preheated separately. The reaction was performed in a temperature-controlled cuvette using a spectrophotometer (Ultrospec 3000, Amersham Bioscience, Freiburg, Germany) by adding enzyme solution to *p*-nitrophenyl-β-D-galactopyranoside solution. The increase of absorbance at 405 nm as result of *p*-nitrophenol release was measured for 2 min. The activity was calculated from the slope of the straight line with absorption coefficients of 0.523 L/mmol/cm (75 °C) and 0.300 L/mmol/cm (37 °C), respectively.

The activity of immobilized CelB was measured by adding immobilized biocatalyst to a preheated substrate solution with a final concentration of 25 mmol/L *p*-nitrophenyl-β-D-galactopyranoside in a closable vial using a thermoshaker (Eppendorf, Hamburg, Germany) for 5 min at 75 °C. To stop the reaction, the vial was put on ice. The absorbance of the clear supernatant was measured photometrically.

One nanokatal is defined as the amount of enzyme that catalyzes the release of 1 nmol of *p*-nitrophenol from *p*-nitrophenyl-β-D-galactopyranoside per second. Total protein concentration was determined according to the method of Bradford (*25*) using bovine serum albumin as a standard.

Determination of Kinetic Parameters. Kinetic parameters (K_M, v_{max}) for CelB (free and immobilized) and *A. oryzae* β-galactosidase were obtained in 1 mL scale from the initial reaction rates at final concentrations of 5−300 mmol/L lactose and lactulose dissolved in acetate buffer (50 mmol/L, pH 5.0), respectively. Enzyme and substrate solutions were preheated at the corresponding reaction temperature separately. The reactions were started by adding enzyme solution or immobilized biocatalyst to substrate solution and incubated in a thermoshaker (Eppendorf) for 5 min at 75 °C (CelB) and 37 °C (β-galactosidase *A. oryzae*), respectively. The reactions were stopped by adding 50 μL of 0.1 mol/L NaOH. The liberated amount of D-galactose was enzymatically determined by a lactose/D-galactose Boehringer kit (R-Biopharm, Darmstadt, Germany). K_M and v_{max} were calculated according to the linearization method of Hanes (*26*).

Biotransformations. Bioconversions of lactose in the presence of fructose using CelB (free and immobilized) and β-galactosidase from *A. oryzae* were performed in acetate buffer (50 mmol/L, pH 5.0) in 1 and 30 mL scales at 75 and 37 °C, respectively.

To investigate the influence of different donor and acceptor concentrations, the initial lactose concentration was varied (0.05, 0.1, 0.2, 0.4, 0.6, 0.8 mol/L), whereas the fructose concentration was kept constant at 1.5 mol/L. In the case of varied fructose concentrations (0.1, 0.5, 0.8, 1.0, 1.5, 2.0 mol/L) the lactose concentration was kept constant at 0.1 mol/L. Conversions were followed until the lactulose concentration was constant or decreasing. Samples from biotransformations were analyzed by TLC and HPLC.

TLC Analysis of Saccharides. TLC analysis was done according to a modified method of Scherz and Bonn (*27*). Aliquots (10 μL) of the reaction mixture were diluted, and 1 μL was spread on a 10 × 10 cm silica gel plate (Merck, Darmstadt, Germany). The solvent system consisted of 2-propanol/acetic acid/0.5% (w/v) boric acid, 60:3:10 (v/v/v). Sugars were visualized by application of aniline−diphenylamine−phosphoric acid solution and heating to 105 °C for ∼5 min. Ten milliliters of a 4% (w/v) solution of anilinium dihydrochloride in ethanol and 10 mL of a 4% (w/v) solution of diphenylamine in ethanol were mixed with 3 mL of 65% (v/v) phosphoric acid. R_f values of the products were compared to R_f values of the standard saccharides (glucose, galactose, fructose, lactose, lactulose).

HPLC Analysis of Saccharides. Samples from bioconversions were analyzed by HPLC to monitor product and substrate concentrations over time. An aliquot (50 μL) of the reaction mixture was added to 950 μL of precooled (4 °C) water (in the case of CelB) or 950 μL of 50 mmol/L NaOH (in the case of *Aspergillus* β-galactosidase) to stop the reaction. Interfering anions were removed by applying the solution (1 mL) to a Strata strong anion exchange column (Phenomenex, Aschaffenburg, Germany), and the sugars were eluted with 1.4 mL of twice-distilled water.

The samples were analyzed by HPLC using a ThermoFinnigan Surveyor autosampler and LC pump (ThermoElectron, Dreieich, Germany) equipped with a 300 × 7.8 mm i.d. Rezex Ca^{2+} column (Phenomenex) at 85 °C. The column was eluted with twice-distilled water at a flow rate of 0.8 mL/min. Sugars were detected with a Sedex 75 evaporative light scattering detector (ELSD) (Sedere, France). Sugar concentrations in the reaction mixtures were determined by external calibration of standard saccharides (glucose, galactose, fructose, lactose, lactulose).

Data Evaluation. All biotransformations and activity measurements including the determination of kinetic constants have been done at least twice in two separate reaction vessels. HPLC and enzyme activity determinations have been done in triplicate. Standard deviations were calculated for each experiment, and error bars representing the standard deviation are included in the figures.

Isolation of Lactulose. For further structural analysis of the main product formed, an aliquot of the reaction mixture of a 30 mL biotransformation using free CelB was semipreparatively purified using a Beckman System Gold HPLC (Munich, Germany) with an ERC-7512 RI detector (Riemerling, Germany) and a 300 × 7.8 mm i.d. Rezex Ca^{2+} column (Phenomenex) eluted with water at 85 °C and a flow rate of 0.8 mL/min. Lactulose-containing fractions were collected and lyophilized.

NMR and LC NMR Analysis. NMR and LC-NMR spectra were recorded on a Varian Unity Inova 500 MHz NMR spectrometer (Darmstadt, Germany). ^{1}H chemical shifts were referenced to the residual solvent signal at δ 4.70 (D_2O) relative to TMS. All 1D (^{1}H, ^{13}C, 1D-TOCSY, DPFGNOE) and 2D NMR (gCOSY, DQFCOSY, TROESY, TOCSY, HOM2DJ, gHSQC, gHSQCTOCSY, CIGAR, gHMQC, g = gradient enhanced) measurements were performed using standard Varian pulse sequences. The spectra were recorded once with 30 mg of lactulose (Sigma-Aldrich, Seelze, Germany) dissolved in 700 μL of D_2O (used for comparison) and twice with 2−3 mg of purified enzymatically produced lactulose.

LC-NMR spectra were recorded in the on-flow mode (24 scans per ^{1}H NMR spectrum) using an ID-PFG probe with a flow cell of 65 μL active volume. Solvent suppression was performed by WET (*28*). The HPLC system consisted of a Varian 9012 pump and a Varian 9050 UV detector (Darmstadt, Germany). An aliquot (~6 mg) of the reaction mixture was separated on a 300 × 7.8 mm i.d. Rezex Ca^{2+} column (Phenomenex) using D_2O as eluent. The system was operated at 85 °C with a flow rate of 0.8 mL/min. Monitoring was performed at 190 nm.

HPLC-MS Analysis. LC-MS analysis was performed using an HPLC pump Pro Star (Varian, Darmstadt, Germany) connected with a TSQ 700 mass spectrometer (Finnigan MAT, San Jose, CA) fitted with an APCI source. Separation of the individual sugars was achieved as described under NMR and LC-NMR Analysis. Despite the fact that the column used cannot be eluted with slightly acidified solvents, acidification was necessary for reproducible negative ion APCI mass spectra of the sugars. It was found that a preconditioning of the mass spectrometer with diluted acetic acid (1% v/v) was sufficient for the reproducible formation of intense $[M - H]^-$ and $[M - H + HAc]^-$ ions for at least 3 h. For the APCI analysis (negative mode) the vaporizer was set to 450 °C and the capillary temperature to 200 °C. Spectra were monitored from *m*/*z* 123 to 600. MS/MS analysis was performed with argon as collision gas at a pressure of 1.8×10^{-3} Torr and −22 eV.

Optical Rotation. Optical rotation was recorded on a Perkin-Elmer 341 polarimeter (Boston, MA). The specific optical rotation $[\alpha]_D^{25}$ −52 (*c* 5.0, H_2O) of purified lactulose was in good agreement with the literature values (*29*) of $[\alpha]_D^{25}$ −50.2.

RESULTS AND DISCUSSION

Kinetic Parameters of CelB and *A. oryzae* β-Galactosidase. Kinetic parameters were investigated to optimize substrate concentrations with regard to maximum reaction rates at minimal biocatalyst concentration. Furthermore, it was important to know whether the formed product lactulose will be a substrate for enzymatic hydrolysis. The kinetic constants K_M and v_{max} were determined for lactose and lactulose hydrolysis, respectively. The results are shown in **Table 1**.

The K_M values of lactulose were lower than for lactose for all three enzyme preparations. Consequently, with decreasing lactose concentration during a biotransformation the formed product lactulose will be hydrolyzed at a certain point. Furthermore, the v_{max} of *A. oryzae* β-galactosidase is higher for both lactose and lactulose compared to CelB. Unlike the free CelB, the immobilized CelB showed much lower v_{max} values.

Table 1. Kinetic Constants for Lactose and Lactulose Hydrolysis by CelB (Free and Immobilized) and *A. oryzae* β-Galactosidase at 75 and 37 °C, Respectively

	lactose		lactulose	
enzyme	K_M[a] (mmol/L)	v_{max}[a] (nkat/mL)	K_M[a] (mmol/L)	v_{max}[a] (nkat/mL)
CelB (free)[b]	38	97	10	25
CelB (immobilized)[b]	30	12	12	8
A. oryzae β-galactosidase (free)[b]	34	133	17	140

[a] Constants were calculated according to the linearization method of Hanes (*26*). [b] Enzyme activity was 65 nkat for all enzyme preparations. Standard deviation < 5%.

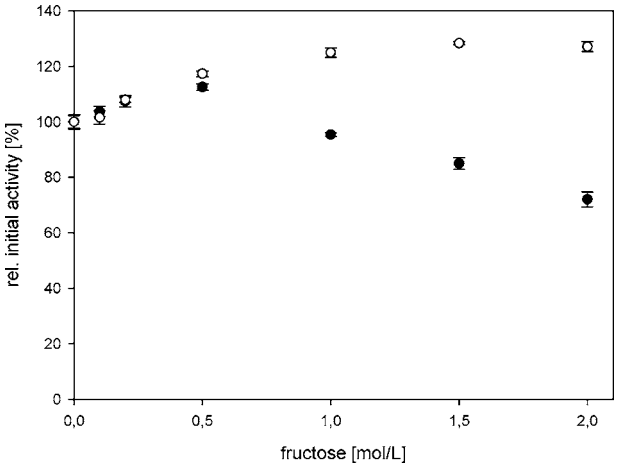

Figure 1. Activation and inhibition effects by D-fructose (0–2 mol/L) on CelB (●, 75 °C) and *A. oryzae* β-galactosidase (○, 37 °C). Activity was measured by the release of *p*-nitrophenol from *p*-nitrophenyl-β-D-galactopyranoside in 1 mL scale. Initial activity (100%) for both enzymes was 65 nkat/mL. Standard deviation < 5%.

Enzyme Activity in the Presence of Different Acceptor Concentrations. CelB is optimally active at about 105 °C and pH 5.0 (*24*). However, this high temperature with the high concentration of reducing sugars (lactose, fructose) induces nonenzymatical browning (Maillard reaction). This effect is much lower at 75 °C, and the residual activity is still ~30%. The *Aspergillus* enzyme has an optimal activity at about 45 °C and pH 5.0, but the operational stability is much higher at 37 °C and the residual activity is still ~50% (*30*). Thus, 75 °C was used for biotransformations with CelB and 37 °C for biotransformations with *A. oryzae* β-galactosidase.

The monosaccharide D-galactose released from lactose is known as a potent inhibitor of many β-galactosidases isolated from mesophilic sources (*31*). CelB as well as *Aspergillus* β-galactosidase are only marginally inhibited by D-galactose ($K_{i,gal,CelB}$ = 300 mmol/L). However, CelB is inhibited competitively by D-glucose ($K_{i,glc,CelB}$ = 76 mmol/L) (*16*). The influence of D-fructose on enzyme activity of CelB and *A. oryzae* β-galactosidase has not been described previously. In the present study the effect of varying the fructose concentration between 0.1 and 2.0 mol/L was investigated (**Figure 1**).

A. oryzae β-galactosidase was activated in the presence of D-fructose in a concentration range of 0.1−1.5 mol/L. At concentrations >1.5 mol/L no further activity augmentation was

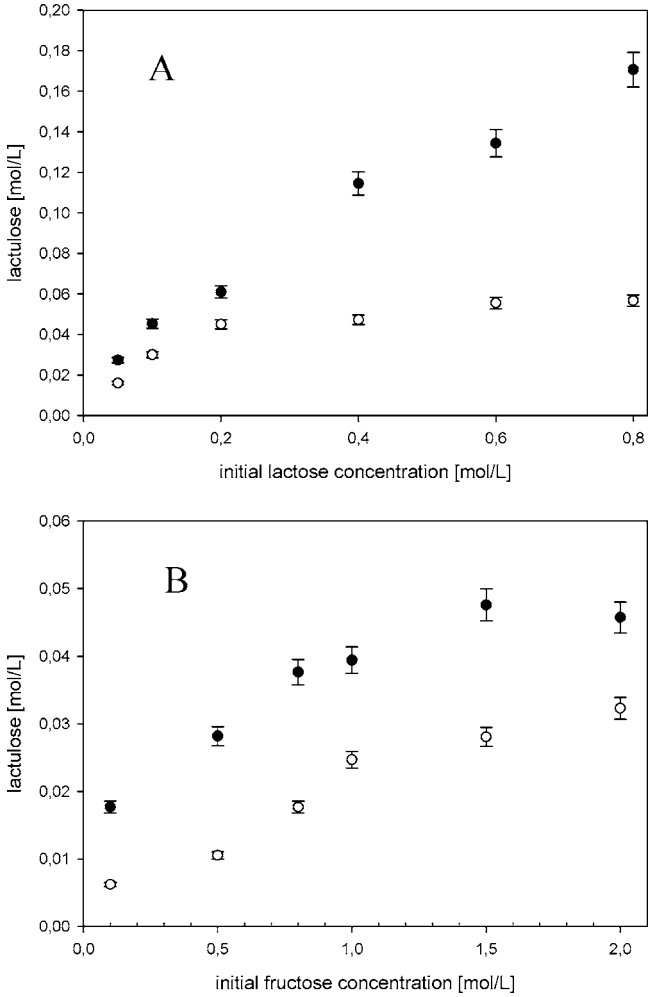

Figure 2. Formation of lactulose as a function of (**A**) initial lactose concentration (1.5 mol/L fructose) and (**B**) initial fructose concentration (0.1 mol/L lactose), respectively. Biotransformations are in 1 mL scale with free CelB (●, 75 °C) and *A. oryzae* β-galactosidase (○, 37 °C) (enzyme activity = 50 nkat/mL). Standard deviation < 5%.

observed. The CelB activity was only slightly increasing until 0.5 mol/L D-fructose, and beyond this concentration a clear inhibition effect was determined.

Influence of Glycosyl Donor and Glycosyl Acceptor Concentration on the Product Yield. The ratio of glycosyl donor to acceptor influences the ratio between hydrolysis and transglycosylation. This was investigated by varying the lactose as well as the fructose concentrations on a 1 mL scale (**Figure 2**). Biotransformations with free enzymes were carried out for 1.5 h, which was the best time period for achieving the highest production yields estimated in prior time course studies using a free glycosidase activity of 50 nkat/mL (data not shown but also confirmed in **Figure 3A**,**B**). The samples (50 μL) of the reaction solution were analyzed by HPLC and TLC.

The absolute product concentration increased with increasing initial lactose concentration (**Figure 2A**), but the relative product yield referring to the initial lactose concentration was decreasing. With increasing initial lactose concentration the formation of oligosaccharides other than lactulose was promoted (data were obtained by TLC, not shown). Thus, higher levels of byproducts were generated with increasing initial lactose concentration. With CelB higher relative product yields (20−55%) were observed than with *A. oryzae* β-galactosidase (7−32%). Maximum relative product yields of 55 and 32%, respectively, were

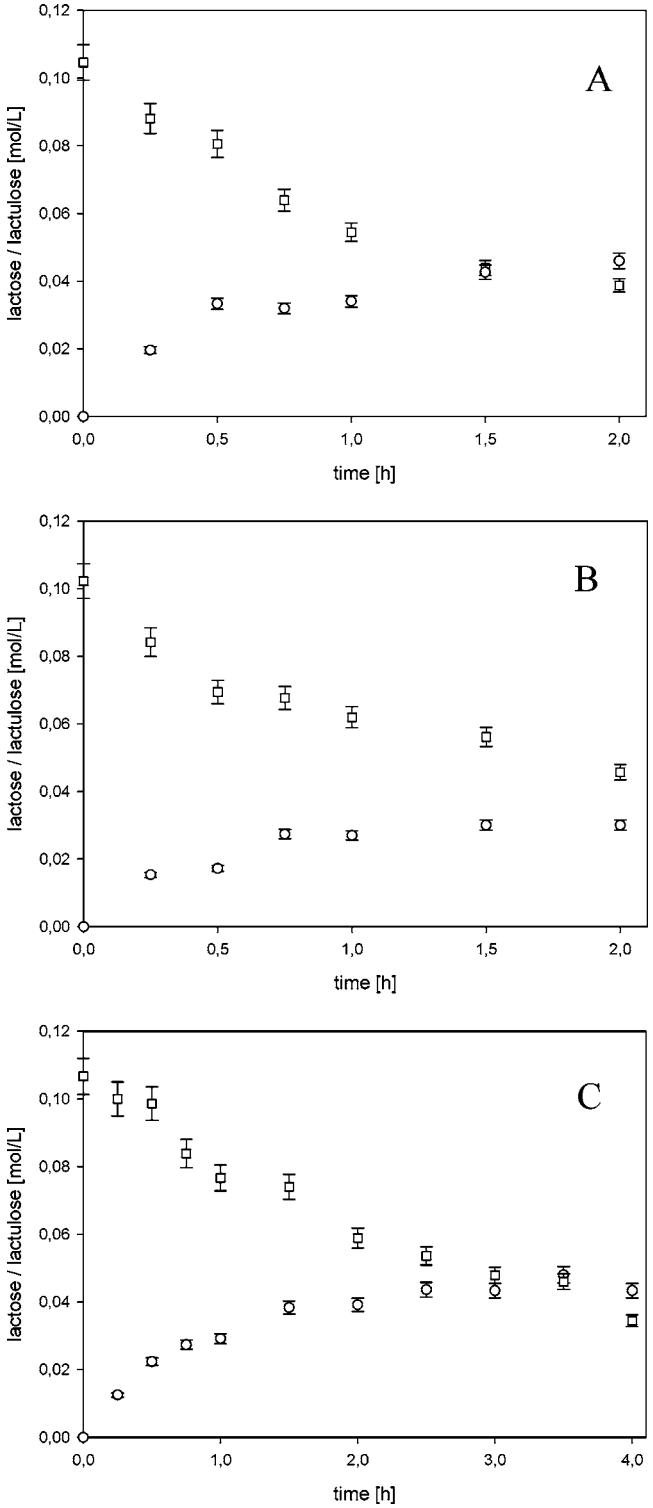

Figure 3. Biotransformation of lactose (0.1 mol/L) in the presence of fructose (1.5 mol/L) in 30 mL scale using CelB free (**A**) at 75 °C, *A. oryzae* β-galactosidase (**B**) at 37 °C, and CelB immobilized (**C**) at 75 °C (lactulose, ○; lactose, □). Enzyme activity was 50 nkat/mL for all enzymes. Standard deviation < 5%.

obtained with an initial lactose concentration of 0.05 mol/L (**Figure 2A**).

In contrast, the fructose concentration was also varied (0.1−2.0 mol/L) and the lactose concentration was fixed at 0.1 mol/L (**Figure 2B**), which seems the best compromise concerning absolute and relative yields of lactulose. Here, both relative yields, the one for the initial fructose concentration and the other

Table 2. Product Yields Relative to Initial Lactose as Well as Initial Fructose Concentration[a]

initial fructose (mol/L)	product yield (%) relative to initial lactose concn[a]		product yield (%) relative to initial fructose concn	
	CelB[c]	*A. oryzae*[c]	CelB[c]	*A. oryzae*[c]
0.10	18	6	18	6
0.50	28	11	6	2
0.80	38	18	5	2
1.00	39	25	4	3
1.50	48	28	3	2
2.00	46	32	2	2

[a] Biotransformation in 1 mL scale with free CelB (75 °C) and *A. oryzae* β-galactosidase (37 °C). [b] Initial lactose concentration = 0.1 mol/L. [c] Enzyme activity was 50 nkat. Standard deviation < 5%.

for initial lactose concentration, were calculated (**Table 2**). The relative product yields referring to the initial fructose concentration decreased from 18 to 2% in the case of CelB and from 6 to 2% in the case of the *A. oryzae* enzyme, whereas the relative product yields referring to the initial lactose concentration increased from 18 to 48% in the case of CelB and from 6 to 32% in the case of the *A. oryzae* β-galactosidase. The lower lactulose yield of 46% (related to lactose) with CelB at a fructose concentration of 2 mol/L can be explained by the inhibition effect of fructose at higher concentrations (**Figure 1**).

In summary, these experiments in which the galactose donor as well as galactose acceptor concentrations were varied showed that in general CelB resulted in higher yields of lactulose compared with *A. oryzae* β-galactosidase. Furthermore, at a low concentration of lactose (0.1 mol/L) the highest lactulose yield (48%) was obtained in the presence of 1.5 mol/L fructose.

Biotransformation of Lactose and Fructose on a 30 mL Scale. The research experiences from the 1 mL scale were transferred to a scale of 30 mL in a small stirred glass reactor. A lactose concentration of 0.1 mol/L and a fructose concentration of 1.5 mol/L were chosen. The results of free CelB and the *Aspergillus* enzyme are shown in **Figure 3A**,**B**. A product yield relative to initial lactose concentration of 44% (46 mmol/L) was reached with free CelB, whereas it was ~30% (30 mmol/L) for *A. oryzae* β-galactosidase. The resulting space-time yields for CelB and *A. oryzae* β-galactosidase were 0.024 and 0.016 mol/L/h, respectively.

Biotransformations in a continuous packed bed reactor for which the CelB has to be immobilized onto a spherical carrier such as Eupergit C are planned in the future. For comparison, a preliminary biotransformation experiment with immobilized CelB onto Eupergit C was performed under the same conditions as for the free enzyme. The obtained relative product yield after 2 h was only 36%, but it was increasing and, a maximum product yield of 45% (47 mmol/L) was obtained after 3.5 h (**Figure 3C**). Thus, the resulting space-time yield for the immobilized CelB (0.012 mol/L/h) is lower than for the free CelB. Nevertheless, the maximum product yield reached was similar to that of the free CelB. Not only both free enzyme preparations but also the immobilized CelB were completely stable during the reaction courses of 2 and 4 h, respectively (residual activity = 100%).

Because of the low K_M values of the three enzyme preparations with lactose (**Table 1**), almost complete lactose hydrolysis was expected. Nevertheless, it was not possible to achieve a lactose concentration below 40 mmol/L under the tested conditions. One reason was probably the product inhibition by the released D-glucose ($K_{i,glc,CelB}$ = 76 mmol/L) or by the formed galacto-oligosaccharides.

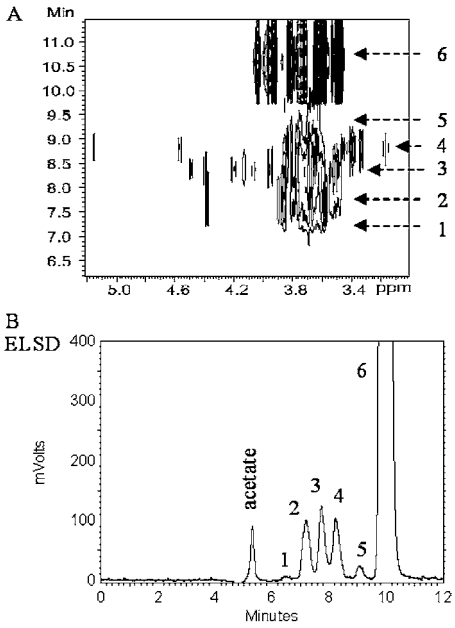

Figure 4. LC-NMR on-flow contour plot (**A**) and HPLC chromatogram of a biotransformation (**B**) of 0.1 mol/L lactose in the presence of 1.5 mol/L D-fructose in a 30 mL scale using free CelB: 1, see text; 2, lactose; 3, lactulose; 4, D-glucose; 5, D-galactose; 6, D-fructose.

Vaheri and Kauppinen (*14*) published a maximum lactulose concentration of ~25 mmol/L (8% of initial lactose concentration) with a space-time yield of 0.019 mol lactulose/L/h at an initial lactose concentration of 0.33 mol/L and a fructose concentration of 1.11 mol/L using the *Saccharomyces fragilis* β-galactosidase. The product concentration reached by Lee et. al. (*13*) was ~42 mmol/L (4% of initial lactose concentration) with a space-time yield of ~0.006 mol lactulose/L/h at an initial lactose concentration of 0.97 mol/L and a fructose concentration of 0.56 mol/L using whole cells of *Kluyveromyces lactis*. From a comparison of these published results with the ones obtained in this study it can be concluded that an optimal ratio of donor to acceptor can increase product yield relative to initial donor concentration. Moreover, maximum product concentrations of ~0.24 mol/L with still acceptable relative product yields of ~30% were reached in our investigations with free CelB using a ratio of donor to acceptor comparable with the one described by Vaheri and Kauppinen (*14*) and Lee et al. (*13*), respectively (**Figure 2A**).

Structural Analysis of the 4-*O*-(β-D-Galactopyranosyl)-D-Fructose Formed. The reaction mixture obtained from the 30 mL scale biotransformation of lactose at 75 °C with free CelB was initially analyzed by LC-MS and LC-NMR to identify the individual compounds without performing time-consuming isolation (**Figure 4**). According to the elution order the major HPLC peaks could be identified as lactose (**2**), D-glucose (**4**), D-galactose (**5**), and D-fructose (**6**) by comparing the NMR and MS data to reference compounds. In the NMR flow cell **2**, **4**, and **5** were obtained as mixtures of their corresponding anomers and **6** was obtained as a mixture of isomers. The on-flow ^{1}H NMR spectrum of the compound corresponding to the minor HPLC peak **1** displayed a large hump in the region of the anomeric protons, which have so far not been attributed to distinct structures. Comparison of the ^{1}H NMR spectrum of compound **3** (HPLC peak 3) with the ^{1}H NMR spectrum of commercial lactulose (**Figure 5**) revealed almost identical peak patterns for both compounds, suggesting that lactulose represents the major enzymatically formed disaccharide.

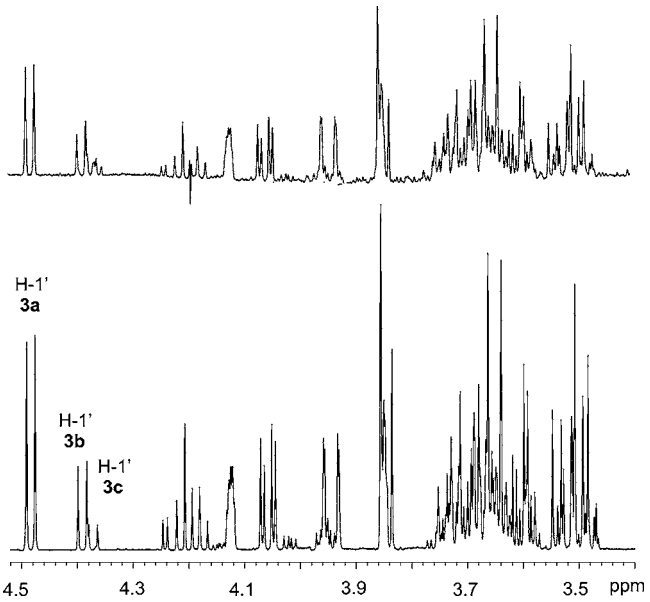

Figure 5. ^{1}H NMR spectra of enzymatically formed lactulose (upper trace) and a reference sample (lower trace) in D_2O at 500 MHz.

To verify the overall structure of lactulose and the position of the glycosidic linkage, lactulose was semipreparatively isolated and submitted to extensive NMR studies. Although ^{13}C NMR data of lactulose data and partially assigned low-field ^{1}H NMR data in aqueous solution have been published (*32*−*34*), to the best of our knowledge there has not been any fully assigned highly resolved ^{1}H NMR spectrum of lactulose. This kind of approach would be of great value to the rapid identification of lactulose in, for example, diluted enzymatical reactions.

The ^{1}H NMR spectrum displayed three β-anomeric galactosyl protons at δ 4.48, 4.39 and 4.37 in a ratio of 66:25:9, indicating an equilibrium mixture of lactulose isomers **3a**, **3b**, and **3c** in D_2O (**Figures 5** and **6**). Complete structure elucidation and full proton assignment of each isomer were performed using 1D (^{1}H, ^{13}C,1D-TOCSY, DPFGNOE) and 2D NMR (gCOSY, DQFCOSY, TROESY, TOCSY, HOM2DJ, gHSQC, gHSQC-TOCSY, CIGAR, gHMQC). HMBC correlations between both H-4 (fructose) and C-1′ (galactose) and between H-1′ (galactose) and C-4 (fructose) unambiguously established the 1 → 4 linkage between the galactose and fructose moieties of each isomer (**Figure 6**; **Table 3**).

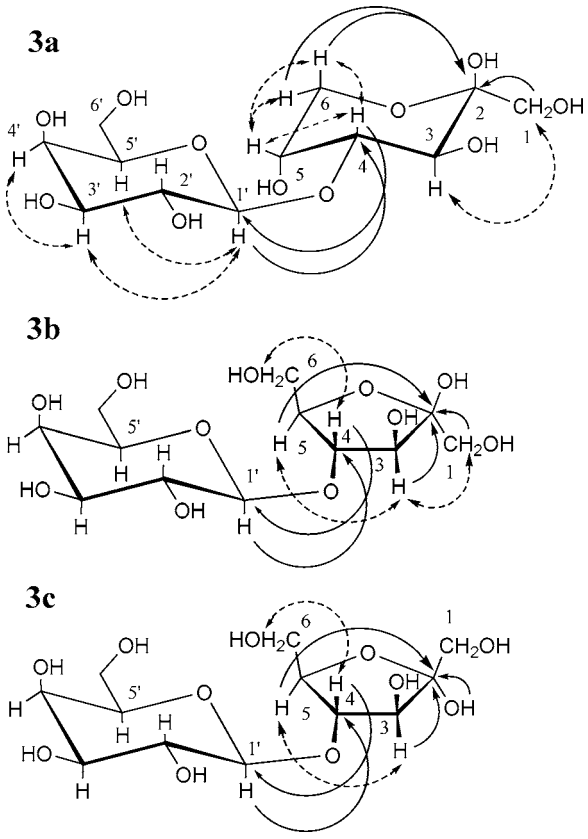

Figure 6. Major isomers of lactulose in D_2O. Arrows indicate important HMBC (→) and ROESY (↔) correlations.

In all cases the $^4C_1$ conformation of the D-galactopyranosyl moiety was deduced from the size of the coupling constants ($^3J_{H-1',H-2'} = 7.8$ Hz, $^3J_{H-2',H-3'} \approx 10$ Hz) and ROEs as well as NOEs between H-1′ and H-3′ and between H-1′ and H-5′. The HMBC correlation between H-6a/b and C-2 of the fructose and a vicinal coupling constant of $J = 10$ Hz between H-3 and H-4 as well as the ROE between H-3 and H-1 established a β-D-fructopyranosyl moiety in the major isomer **3a** (66%). HMBC correlations between H-5 and C-2 of the fructose indicated furanoside structures for the remaining two main

Table 3. ^{1}H NMR and ^{13}C NMR Data of the Major Isomers of Lactulose

	^{1}H NMR, δ, mult, J (Hz)[a]			^{13}C NMR, δ[b]		
position	**3a**	**3b**	**3c**	**3a**	**3b**	**3c**
1a/b	3.65, d, 11.8 3.49, d, 11.8	3.52, d, 12.2 3.48, d, 12.2	3.59, s	64.08	62.67	62.96
2				98.26	102.85	105.14
3	3.84, d, 10.0	4.22, d, 7.3	4.24, d, 4.2	66.23	74.87	80.92
4	4.06, dd, 3.4, 9.9	4.19, dd, 6.5, 7.3	4.02, dd, 4.1, 7.5	77.59	84.34	85.46
5	4.13, dt, 3.5,1.7	3.95, ddd, 3.5, 5.6, 6.7	4.14, ddd, 3.4, 5.3, 8.2	66.97	80.24	80.90
6a/b	3.94, dd, 1.4, 12.9 3.68, dd, 2.0, 13.0	3.73, dd, 3.7, 12.3 3.64, dd, 5.8, 12.3	3.75, dd, 3.1, 12.4 3.66, dd, 4.9, 12.4	63.18	62.82	61.35
1′	4.48, d, 7.8	4.39, d, 7.8	4.37, d, 7.8	101.02	103.05	103.45
2′	3.53, dd, 7.8, 10.0	3.49, dd, 7.5, 9.8	3.48, overlapped	70.95	70.90	70.84
3′	3.61, dd, 3.7, 9.9	3.59, dd, 3.7, 10.0	3.58, dd, 3.4, 10.0	72.79	72.73	72.69
4′	3.85, bdd, 1.0, 3.4	3.84, bd, 2.0	3.84, overlapped	68.88	68.67	68.71
5′	3.64, ddd, 0.9, 3.8, 8.5	3.66, bdd, 3.7, 8.8	3.65, overlapped	75.55	75.46	75.50
6′a/b	3.73, dd, 8.3, 11.8 3.67, dd, 3.7, 11.8	3.71, overlapped 3.69, overlapped	*c*	61.34	61.23	*c*

[a] Observed coupling constants were not averaged. Assignments are based on gCOSY, DQFCOSY, 1D-TOCSY, DPFGNOE, TROESY, HOM2DJ, CIGAR, gHSQCTOCSY, gHSQC, and gHMQC spectra. D_2O, ref = 4.7 ppm; $J = 25$ °C; 500 MHz. [b] ^{13}C NMR at 125.90 MHz. [c] Due to extremely overlapping signals ^{1}H and ^{13}C chemical shifts could not be assigned unambiguously.

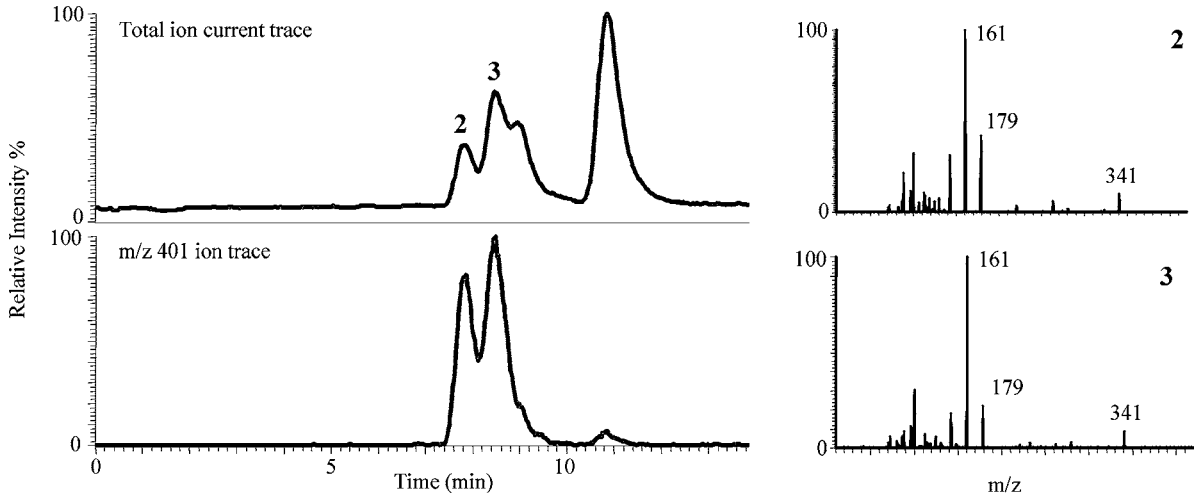

Figure 7. APCI-HPLC-MS total ion current and ion trace of $[M - H + HOAc]^-$ *m*/*z* 401 with corresponding APCI-HPLC-MS/MS of *m*/*z* 401 from lactose (**2**) and lactulose (**3**).

isomers **3b** and **3c.** Further analysis of the 1D and 2D NMR spectra confirmed the presence of two D-fructofuranose isomers differing only in the stereochemistry of the anomeric carbon C-2. A strong ROE between H-1 and H-3 established the second main isomer (25%) as 4-*O*-(β-D-galactopyranosyl)-β-D-fructofuranose, **3b**. From these findings it can be concluded that an aqueous solution of lactulose mainly consists of three of five possible isomers: 4-*O*-(β-D-galactopyranosyl)-β-D-fructopyranose (**3a**, 66%), 4-*O*-(β-D-galactopyranosyl)-β-D-fructofuranose (**3b**, 25%), and 4-*O*-(β-D-galactopyranosyl)-α-D-fructofuranose (**3c**, 9%) (**Figure 6**). This corroborates the results of Pfeffer and Hicks (*32*) and Jeffrey et al. (*29*) deduced from ^{13}C NMR. At a concentration of 30 mg of lactulose in 700 μL of D_2O no NMR spectroscopic evidence could be found for the two remaining isomers, 4-*O*-(β-D-galactopyranosyl)-α-D-fructopyranose and the open-chain form of the fructose moiety. Nevertheless, Pfeffer and Hicks (*32*) observed the open-chain form in very low concentration by using more highly concentrated solutions of lactulose (200−250 mg) in their NMR studies. The specific optical rotation of purified lactulose was in good agreement with literature values (*29*).

HPLC-MS of the above reaction mixture showed the $[M - H]^-$ at *m*/*z* 341 and a strong $[M - H + HOAc]^-$ signal at *m*/*z* 401 for the HPLC peaks 2 and 3 (**Figures 4B** and **Figure 7**). MS/MS experiments on the more intense adduct ions at *m*/*z* 401 resulted in almost identical fragmentation patterns for compounds **2** and **3** (**Figure 7**). A significant difference could be observed for the intensity of the peak at *m*/*z* 179 (40% in the case of **2** and 20% in the case of **3**). Comparison of these findings with MS/MS data of reference compounds revealed that these different intensities are indicative for lactose (**2**) and lactulose (**3**).

In enzymatic transglycosylation reactions primary hydroxyl groups will be glycosylated preferentially. To overcome this fact many researchers have used aglycons with protected primary hydroxyl groups for transglycosylation reactions with different glycosidases (*35*, *36*). Nevertheless, in most cases a mixture of regioisomers is obtained. Petzelbauer et al. (*21*) showed that during galacto-oligosaccharide synthesis by CelB at high lactose concentrations (0.8 mol/L) the preferred newly formed glycosidic bonds are β(1→3) and β(1→6). In the second course of the reaction an accumulation of 6-*O*-(β-D-galactopyranosyl)-D-glucose at the end of lactose hydrolysis reflects a 3−10-fold specificity of CelB for the hydrolysis of β(1→3)- over β(1→6)-linked glucosides. The transgalactosylation from lactose to fructose with CelB in the present study resulted in one regioisomer in high excess (>95%), whereas other oligosaccharides were generated in minor amounts. The reaction was regioselective with respect to the secondary C-4 hydroxyl group of fructose, resulting in 4-*O*-(β-D-galactopyranosyl)-D-fructose.

Conclusion. The results of the present study demonstrate the enzymatic production of lactulose with CelB in yields of 16 g/L without any loss of enzyme activity. By NMR studies it could be unambiguously established that the major formed product with CelB was 4-*O*-(β-D-galactopyranosyl)-D-fructose. In future investigations the immobilized enzyme, which performed as well as the free CelB in these studies, will be used for continued biotransformations in a packed-bed reactor.

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