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Synthesis of UDP-activated Oligosaccharides with Commercial β-galactosidase from *Bacillus circulans* under Microwave Irradiation

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Abstract

We report here on the synthesis of nucleotide activated oligosaccharides by transglycosylation with β-galactosidase from *Bacillus circulans* applying microwave irradiation (MWI)[1] and conventional heating. The presented products could serve as novel inhibitors or donor substrates of Leloir-glycosyltransferases. Some of them have been isolated from human milk but the biological role remains unclear due to limited access to the nucleotide oligosaccharides. The synthesis with β-galactosidases is challenging, because of competing hydrolysis of the product by the same enzyme. Effects of MWI and thermal heating on the hydrolytic and synthetic performance of the enzyme were systematically analysed and described here. We demonstrate that under both conditions similar product yields are obtained, however, the enzymatic hydrolysis of the product is significantly decreased under MWI leading to stable product formation. The obtained product yields and absence of product hydrolysis under MWI can be rationalized by time-dependent activation and inactivation of the β-galactosidase.

Keywords

Bacillus circulans; β-galactosidase; microwave; transglycosylation; nucleotide oligosaccharides

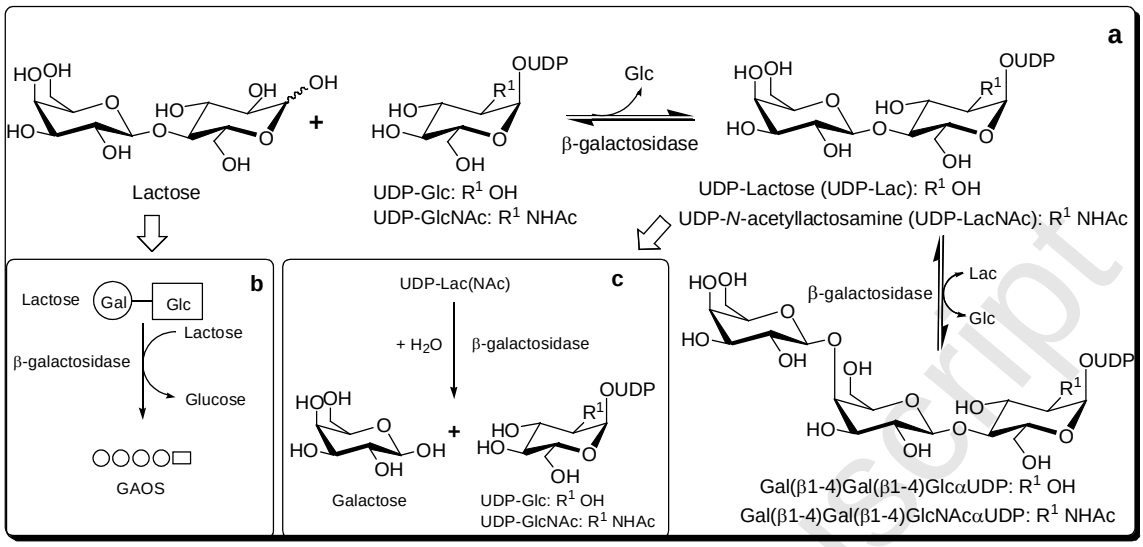
Graphical abstract

[1] MWI : microwave irradiation

1. Introduction

UDP-activated oligosaccharides, like UDP-LacNAc (Gal(β1-4)GlcNAcα1-UDP) and Fuc(α1-2/4)Gal(β1-4)GlcNAc-UDP, were originally identified as glycoconjugates in human milk and colostrum [1-4]. Their biosynthesis and biological role are still unknown. They could possibly serve as inhibitors or substrates of Leloir-glycosyltransferases. The latter would open an attractive strategy for the *in vitro* synthesis of oligomeric glycan structures once the donor substrates can be synthesised for the screening of glycosyltransferases adding two or more sugar units in one catalytic step. Access to UDP-activated oligosaccharides has been demonstrated by us and others using biocatalytic [5-8] and chemical methods [9], respectively. The stereo- and regioselective synthesis of UDP-Lactose (Gal(β1-4)Glcα1-UDP) and UDP-LacNAc (Gal(β1-4)GlcNAcα1-UDP) was accomplished with a mesophilic β-galactosidase from *Bacillus circulans* (Biolacta N5™, Daiwa Kasei KK, Osaka, Japan) using a kinetic approach for the transglycosylation reaction with lactose and UDP-glucose or UDP-GlcNAc, respectively [8]. Further transglycosylation of galactose from lactose onto the UDP-disaccharides yielded also UDP-trisaccharides (Scheme 1a).

Besides the formation of UDP-Lac(NAc) and -oligosaccharides enzymatic hydrolysis of lactose is accompanied by a general galactosyl transfer onto remaining lactose, thereby producing galacto-oligosaccharides (GAOS) (Scheme 1b). GAOS are prebiotics or bifidogenic factors, promoting the growth of beneficial intestinal bacteria like *Bifidobacteria* and *Lactobacilli* [10]. These compounds are non-digestible carbohydrates with health-promoting and disease-preventing properties in humans and known as functional food [11]. The synthesis of individual compounds belonging to the group of human milk oligosaccharides is therefore of general interest.



Scheme 1. Synthesis of UDP-Lac(NAc) and galacto-oligosaccharides (GAOS) by β-galactosidase from *Bacillus circulans*. Kinetically controlled transglycosylation reactions for the synthesis of UDP-Lac(NAc) (**a**) and GAOS **(b)**. Competitive enzymatic product hydrolysis of UDP-Lac(NAc) due to enzymatic hydrolysis (**c**).

The main drawback of synthetic reactions with β-galactosidases is that the equilibrium greatly favours hydrolytic over synthetic reactions in aqueous systems, which leads to low product yields due to enzymatic product hydrolysis [12] (Scheme 1c). A common strategy for reaction engineering of transglycosylation reactions is to reduce the water content in the reaction mixture. High substrate concentrations, the addition of organic solvents, and the presence of ionic liquids result in higher product yields [13,14]. We accomplished the synthesis of nucleotide activated oligosaccharides in frozen aqueous solutions at -5 °C with constant product yields over time [8]. However, the elongated reaction time in the range of days is a drawback of enzymatic synthesis in frozen solution [8]. Therefore, further strategies are needed for the synthesis of glycoconjugates by transglycosylation which give constant product yields at ambient temperature and moderate reaction time.

Microwave assisted synthesis has become a frequently employed method in organic chemistry. Microwave irradiation (MWI) has been demonstrated to significantly increase rates and yields of organic and inorganic reactions [15]. The influence of MWI on biocatalysis has been investigated for reactions employing lipases and glycosidases [16,17]. In contrast to conventional heating increased reaction rates and product yields, concomitantly with lowered product hydrolysis were observed even at modest temperature by MWI. The so called specific effect of MWI is due to the direct interaction of microwaves with highly polar reagents and polar regions of the catalysts in the reaction mixture which align with an external oscillating field due to their high dipole moments [18]. It has been demonstrated, that MWI is

mandatory for some biocatalytic reactions, which are not amenable by thermal heating alone. An example is the activation of CelB, a β-glucosidase from the hyperthermophilic archaeon *Pyrococcus furiosus*. The enzyme could strongly be increased towards the hydrolytic reaction only based on MWI, but not on a temperature effect [19]. Transglycosylation reactions with a hyperthermophilic enzyme from *Sulfolobus solfataricus* onto simple alcohols were found to have enhanced kinetics and reduced product hydrolysis when performed in dry media under microwave irradiation [16]. Immobilized β-galactosidase from *Kluyveromyces lactis* showed a 217-fold increase in GAOS formation with low product hydrolysis under MWI in the presence of hexanol [20]. These examples demonstrate that MWI can have beneficial effects on transglycosylation reactions with glycosidases from different sources.

We here present our work on the synthesis of nucleotide activated oligosaccharides with commercial β-galactosidase from *Bacillus circulans* under focused MWI in comparison with conventional heating at 30 °C. In our previous studies moderate product yields without product hydrolysis were only obtained when transglycosylation reactions were conducted in frozen aqueous solutions at -5 °C with reaction times over several days [8]. Focused MWI improves the reaction performance at 30 °C with shorter reaction times.

2. Experimental

2.1. Biological and chemical materials

A soluble commercial preparation of β-galactosidase from *Bacillus circulans*, available as "BIOLACTASA NTL" (Biocon, Spain), with an activity of 3409 U/mL, was used. UDP-glucose (UDP-Glc), *p*-nitrophenole (*p*NP) and *p*-nitrophenyl-β-D-galactopyranoside (*p*NPG) were obtained from Sigma, D-lactose monohydrate was from Toronto Research Chemicals Inc., Gal(β1-3)GlcNAc and Gal(β1-6)GlcNAc were obtained from Dextra (UK), Gal(β1-4)GlcNAc from Carbosynth (UK), Citric acid monohydrate was purchased from Fluka, di-sodium hydrogen phosphate and all other chemicals were from Roth. Dowex 1x2 100-200 (CL) resin material for ion exchange (IEX) chromatography was from DOW Water Solutions (Dow Europe, Edegem, Belgium). Sephadex G10 from GE-Healthcare (Munich, Germany) was used for desalting of products.

2.2. Microwave equipment and irradiation control

The CEM Focused Microwave™ Synthesis System, Model Discover, with a continuous microwave power delivery system programmable between 0 - 300 Watt (+/- 30 Watt) and an "invasive" fibre optic temperature control system (programmable from -80 ºC to 250 ºC) was used. In order to effectively cool the reaction mixture to ambient temperatures during irradiation a jacketed reaction vessel with cryogenic cooling, available as CEM CoolMate™ Accessory System, was employed. An external cryostat (Julabo, Seelbach, Germany) was connected to the CEM CoolMate™ Accessory System for the control of the vessel temperature. The intensity of irradiation could be varied by differences between the cooling temperature and the given synthesis temperature in the reaction vessel. Thereby, an indirect control of the irradiation intensity via the cooling temperature was possible.

2.3. Enzyme activity assay

The BIOLACTASA NTL enzyme solution with a protein content of 32.5 mg/mL was diluted $1:10^4$ in Milli Q H_2O and stored on ice until the activity assay was performed. The reaction mixture contained 10 µL of diluted enzyme solution which was added to 90 µL of a 4 mM *p*NPG in 50 mM citrate-Na_2HPO_4-buffer, pH 6.0, and incubated at 37 °C for 5 minutes. The reaction was stopped by the addition of 200 µL 0.2 M Na_2CO_3. The released *p*NP was quantified in a spectrophotometer (Synergy 2, BioTek., Bad Friedrichshall, Germany) at 405

nm using external calibration with *p*NP. Results were reported in units, where one unit is defined as the amount of enzyme which releases 1 µmol *p*NP from *p*NPG per minute. As a negative control 10 µL Milli Q H_2O were added to the reaction mixture instead of enzyme. No release of free *p*NP could be observed. Residual activity after conventional heating or microwave irradiation of enzyme solutions was determined by collecting enzyme aliquots which were then diluted to $1:10^4$ in ice cold Milli Q H_2O and assayed as described (see supporting information on section 1.1.).

2.4. Specificity of β-galactosidase from Bacillus circulans

The disaccharides (5 mM) Gal(β1-3)GlcNAc, Gal(β1-4)GlcNAc and Gal(β1-6)GlcNAc were tested for hydrolysis by β-galactosidase (6.8 U/mL) in 50 mM citrate-Na_2HPO_4-buffer, pH 4.5 at 30 °C. Aliquots of the reaction solution were collected and denatured at 95 °C for 5 minutes. Percentage hydrolysis of the disaccharides was calculated from the peak area of the products in HPAEC-PAD analysis (for results see supporting information on section 1.2.).

2.5. Hydrolytic activity under MWI

The substrate solution (270 µL of 4 mM *p*NPG in 50 mM citrate-Na_2HPO_4-buffer, pH 4.5) was cooled down to 8 °C. The enzyme solution (30 µL with a protein content of 32.5 mg/mL) was added and the assay was exposed to a temperature gradient to reach 20 °C within 60 seconds by microwave irradiation or by conventional heating, respectively (see also supporting information on section 1.3.). The reaction was then stopped by adding 600 µL of 200 mM Na_2CO_3 and the released *p*NP was detected at 405 nm in a spectrophotometer (Synergy 2, BioTek., Bad Friedrichshall, Germany). A control experiment without enzyme was performed to determine the amount of *p*NP released by chemical substrate hydrolysis under microwave irradiation or conventional heating.

2.6. Transglycosylation

Synthetic reactions under MWI were carried out in the CEM Discover® CoolMate system at 30 °C and ~ 20 Watt. The reaction solution (600 µL for analytical and 2400 µL for preparative scale) contained a lactose concentration of 500 mM in 50 mM citrate-Na_2HPO_4-buffer, pH 4.5. For the production of nucleotide activated oligosaccharides 100 mM UDP-Glc or UDP-GlcNAc solutions, respectively, where added as acceptor substrates. The reaction solution was preheated by conventional heating or MWI to reach 30 °C (measured by fibre

optics sensor), followed by the addition of β-galactosidase, reaching a starting activity of 680 U/mL. Aliquots of the reaction solution were collected. The enzyme was denatured at 95 °C for 5 minutes. The formation of UDP-oligosaccharides and GAOS was detected by capillary electrophoresis (CE) or HPAEC-PAD analysis, respectively, as described below (see also supporting information on section 1.4).

2.7. Isolation of UDP-activated oligosaccharides

Product isolation was performed as described previously [6,7]: The enzyme was separated from the reaction mixture by denaturation at 95 °C for 5 minutes followed by centrifugation. The nucleotide-activated sugars in the supernatant were separated from the neutral sugars and buffer salts by anion-exchange chromatography on a Dowex 1x2, 100-200 mesh, Cl^--form resin (DOW Water Solutions, Dow Europe, Edegem, Belgium, 450 mL gel bed volume) in a XK 50/60 column (GE-Healthcare, Munich, Germany). 6 mL were loaded onto the column equilibrated with Milli Q H_2O. After a washing step with Milli Q H_2O elution was achieved by a salt gradient (0-1 M NaCl, 2 L) and a flow rate of 6 mL/min. Fractions (8 mL) containing nucleotide sugars (UV detection at 254 nm) were pooled and concentrated *in vacuo* to 10 mL. An equal volume of ice-cold ethanol was added and precipitated NaCl removed by centrifugation. The ethanol in the supernatant was evaporated *in vacuo*. The remaining salt was removed by gel filtration on a Sephadex G10 resin (1750 mL, XK 50/100 column, (GE-Healthcare, Munich, Germany): The sample (8 mL) was loaded onto the water-equilibrated column and eluted with Milli Q H_2O at a flow rate of 6 mL/min. Nucleotide sugar containing fractions (UV detection at 254 nm) were pooled and concentrated *in vacuo* to 10 mL.

2.8. Capillary electrophoresis (CE-UV)

Samples containing UDP-saccharides were diluted $1:10^2$ in Milli Q H_2O and analysed by capillary electrophoresis (CE) using a P/ACE MDQ apparatus from Beckman Coulter (Krefeld, Germany), equipped with a variable UV detector set to 254 nm. Separation of NDP-sugars, NDP, NMP and nucleosides was accomplished with an untreated fused-silica capillary (I.D. 75 µm, 60.2 cm total capillary length, 50 cm to the detector, Polymicro Technologies, Phoenix, Arizona, USA) using borate buffer (50 mM sodium tetraborate/64 mM borate, pH 9.0) or NH_4OAc solution (pH 9.2, 50 mM) at 25 kV and 25 °C. Capillary conditioning was performed by flushing the capillary with 0.1 M sodium hydroxide solution for 2 minutes, with

water for 2 minutes and with running buffer (50 mM sodium tetraborate/64 mM borate, pH 9.0 or NH_4OAc solution, pH 9.2, 50 mM) for 2 minutes. Samples were injected by pressure (5 seconds at 0.5 psi in the forward direction) and separated within 30 minutes (borate method) or 15 minutes (NH_4OAc method) total migration time. Before each run, capillary reconditioning was achieved by flushing with NaOH, water and running buffer for 2 minutes.

2.9. CE-MS analysis

Separation of the nucleotide sugar fraction was performed with a capillary electrophoresis system 7100 (Agilent, Waldbronn, Germany) using bare fused silica capillaries (50 µm i.d., length 60 cm; Polymicro Technologies, Phoenix, Arizona, USA). Concentrations of nucleotide sugars in the samples were estimated from the electropherograms by peak areas. Based on an UDP-GlcNAc calibration curve, concentrations of UDP-GlcNAc and the *N*-acetylated UDP-disaccharide and UDP-trisaccharide in the sample were estimated to be 0.53; 0.09 and 0.03 mM, respectively, before dilution with running buffer. Concentrations of UDP-Glc, UDP-disaccharide and UDP-trisaccharide in the second sample were estimated via a UDP-Glc calibration curve and estimated to be 0.14; 0.15 and 0.05 mM, respectively, before dilution with running buffer. Capillary conditioning was performed by flushing the capillary with 1 M sodium hydroxide solution for 20 minutes and with water for 10 minutes. A NH_4OAc solution (pH 9.2, 50 mM) was used as running buffer, injection of the isolated nucleotide sugar fraction diluted 1:5 with running buffer was performed hydrodynamically (5 seconds at 50 mbar). A separation voltage of 25 kV was used. Before each run, capillary reconditioning was achieved by flushing with running buffer for 5 minutes. Mass spectrometric detection was performed on a 6520 accurate-mass quadrupole time-of-flight (qToF) mass spectrometer from Agilent (Waldbronn, Germany), which was directly coupled to the CE-system via a coaxial sheath liquid electrospray interface. The sheath liquid (a 1:1 mixture of H_2O : isopropanol containing 1 % acetic acid) was used at a flow rate of 4 µL/min. The qToF was operated with an electrospray potential of 4 kV, a nebulizer gas stream of 5 psig and a drying gas stream of 4 L/min with a temperature of 300 °C. The fragmentor voltage was set to 215 V in order to ensure maximum signal intensity. The analytes were detected in the negative-ion mode in the m/z-range of 100-1700, with external mass calibration.

2.10. High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD)

HPAEC-PAD was employed for the analysis of formed GAOS and for monosaccharide analysis using a Dionex system (Dionex, Calif., U.S.A.) equipped with an electrochemical detector (Model ED40) and a gold electrode. GAOS containing samples were diluted $1{:}10^5$ in Milli Q H_2O. Samples for monosaccharide analysis from hydrolysis of disaccharides were diluted $1{:}10^3$ in Milli Q H_2O. Aliquots of samples (250 µL) were injected by an autosampler (HPLC 360 autosampler, Kontron Instruments) into a CarboPac PA-1 column (250×4 mm i.d., Dionex and Guard column 4×50 mm; Dionex). The mobile phase consisted of 100 mM NaOH solution with isocratic elution at a flow rate of 1 mL/min. The working potential was 0.05 V and the total retention time 30 minutes.

3. Results and discussion

First experiments aimed for the detection of the specific effect of microwave irradiation on the hydrolysis of *p*NPG by β-galactosidase from *Bacillus circulans*. We then studied the transglycosylation reaction for the synthesis of nucleotide oligosaccharides under MWI in comparison to conventional thermal heating. Finally, the influence of MWI on the long term performance of the enzyme was characterised and compared for identical conditions under conventional thermal heating. The identity of UDP-activated oligosaccharides was confirmed by CE-MS analysis. The regiospecificity of the enzyme was deduced from its ability to hydrolyse the disaccharides Gal(β1-3)GlcNAc, Gal(β1-4)GlcNAc, and Gal(β1-6)GlcNAc. The disaccharide Gal(β1-4)GlcNAc was rapidly hydrolysed, whereas the β6-linked galactose was only slightly removed from the disaccharide. The Gal(β1-3)GlcNAc disaccharide was not cleaved (Fig. S1 supporting information). Our results are in accordance with previous studies demonstrating that the regioselectivity of transglycosylation reactions is highly dependent on the specific hydrolysis of glycosidic bonds [21]. Moreover, studies on the synthesis of *N*-acetyllactosamine confirmed the regioselective transglycosylation with other commercial preparations of β1-4-galactosidase from *Bacillus circulans* [22,23]. Our own studies demonstrated the formation of β1-4 linked UDP-di- and trisaccharides with β-galactosidase from *Bacillus circulans* [8]. We concluded therefore that the hydrolytic regioselectivity of the β-galactosidase from *Bacillus* circulans (BIOLACTASA NTL) reflects also its regioselectivity in the formation of transglycosylation products.

3.1. Effect of MWI on enzymatic hydrolysis of pNPG

The specific effect of microwave irradiation on biocatalysis enables reactions, which cannot proceed by thermal heating alone. The verification for this proposed MWI effect has recently been demonstrated for β-glucosidase from the hyperthermophilic archaeon *Pyrococcus furiosus* [19]. The activity of the *Pyrococcus* enzyme was determined under thermal heating and MWI at temperatures far below the optimum of the hyperthermophilic enzyme. In the absence of MWI no significant enzyme activity was detected. In contrast, with MWI at 300 Watt and the same reaction temperature the enzyme showed a high increase in activity demonstrating that MWI can trigger high biocatalytic rates of hyperthermophilic enzymes at low temperatures.

We investigated the effect of MWI on the mesophilic β-galactosidase from *Bacillus circulans* using the same conditions: online temperature control and reaction temperature far

below the temperature optimum of the enzyme. The hydrolysis of *p*NPG by "BIOLACTASA NTL" was measured for MWI and thermal heating performing a temperature gradient in the range of 8-20 °C for 60 seconds which is far below the optimum temperature. The optimum temperature is 65 °C for this mesophilic enzyme (information of the supplier). The temperature gradients for thermal heating and MWI were very similar (see supporting information Fig. S2). Within the indicated temperature gradient from 8 °C to 20 °C in 60 seconds mesophilic β-galactosidase formed twice more *p*NP under MWI (Fig. 1). Hydrolysis of *p*NPG under MWI without enzyme was only 0.02 %, revealing that MWI does have a direct effect on the biocatalyst and is not responsible of the cleavage of the glycosidic bond itself.

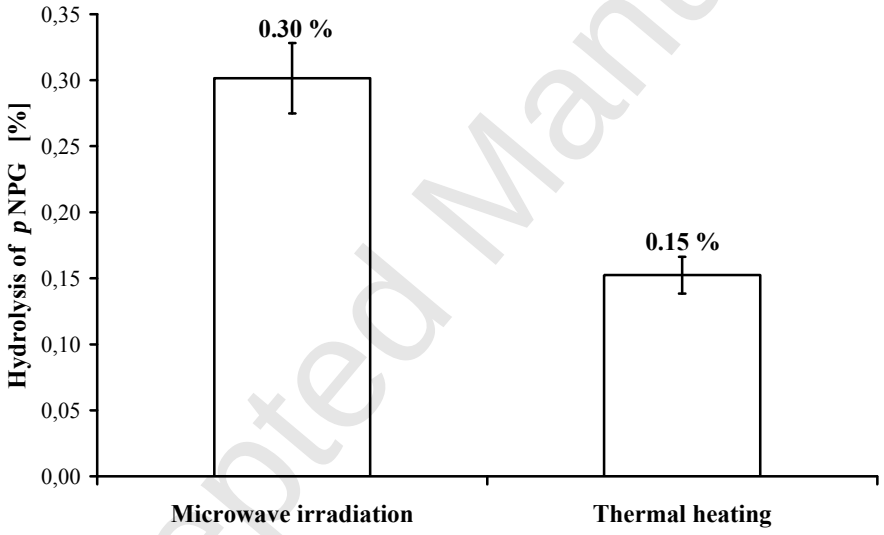


Fig. 1. Effect of MWI on hydrolysis of *p*NPG by β-galactosidase from *Bacillus circulans.*

3.2. Transglycosylation reactions under MWI

Encouraged by these results we further investigated whether MWI has a similar "activation effect" on the transglycosylation reaction at 30 °C and can thus replace the slow reaction at -5 °C [8] with suppression of product hydrolysis. *Bacillus circulans* β-galactosidase in particular is well known for excellent transglycosylation activity [23-26]. At high substrate concentrations β-galactosidases catalyse the transglycosylation reaction with transfer of galactose to an acceptor molecule like UDP-Glc(NAc) producing nucleotide activated oligosaccharides (Scheme 1a) or to lactose producing GAOS (Scheme 1b).

Experiments with 100 mM UDP-Glc as acceptor substrate and 500 mM lactose as donor substrate were performed to produce UDP-activated oligosaccharides at 30 °C under thermal heating (TH) or MWI at ~ 20 Watt, respectively. With CE-UV analysis the formation of UDP-oligosaccharides up to a chain length of four sugar residues was detected (see supporting information Fig. S3) and signals identified by detailed analysis by CE-MS (see 3.4). Fig. 2 depicts the sum of UDP-activated oligosaccharides for the reaction under TH and MWI, respectively. The initial reaction rates are comparable under both conditions; however, product yield (24 %) is constant under MWI due to lack of hydrolysis of UDP-activated oligosaccharides. With TH product hydrolysis starts already after 5 minutes incubation time whereas product yield remains constant with MWI over an incubation period of 90 minutes. We concluded that MWI minimizes product loss by hydrolysis at ambient temperatures.

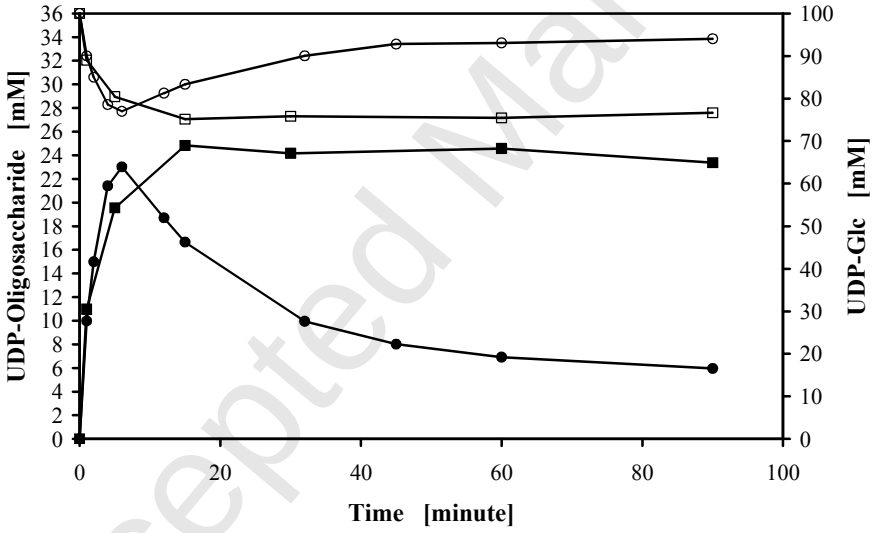


Fig. 2. Comparison of formation of UDP-activated oligosaccharides at 30 °C with β-galactosidase from *B. circulans* under thermal heating (TH) and ~ 20 Watt microwave irradiation (MWI). UDP-oligosaccharides were analysed with CE and plotted as the sum of all products. Products: UDP-activated oligosaccharides under TH ● and under MWI ■. Substrate: UDP-Glc under TH ○ and under MWI □.

The time course of the formation of the main UDP-oligosaccharides clearly demonstrates that the concentrations of single products remain stable under MWI (Fig. 3A). UDP-disaccharide is the main product after 15 minutes reaction time followed by the UDP-tri- and tetrasaccharide (see also supporting information Fig. S4). This product composition remains stable even after 90 minutes reaction time. In contrast, with thermal heating UDP-disaccharide formation is highest after 5 minutes and decreases rapidly due to the formation

of UDP-trisaccharide and enzymatic hydrolysis with formation of UDP-Glc (Fig. 3B). After 90 minutes reaction time the UDP-trisaccharide remains the single product with a low yield (see also supporting information Fig. S4). These results emphasise that reaction control is by far more difficult under thermal heating. Suppression of product hydrolysis was also observed for the synthesis of UDP-oligosaccharides at -5 °C due to the reduced water content in frozen solutions and the reduced activity of β-galactosidase [8]. However, products were only obtained after three days of incubation. In contrast, MWI reactions are performed at 30 °C with stable product concentrations after 15 minutes incubation time.

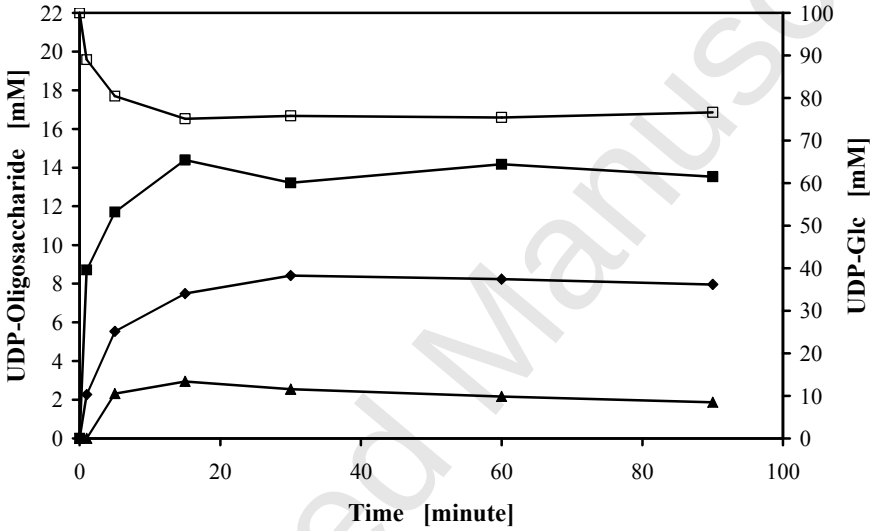


Fig. 3 A: Formation of UDP-activated oligosaccharides at 30 °C with β-galactosidase from *B. circulans* under ~ 20 Watt microwave irradiation (MWI). Products: UDP-disaccharide ■, UDP-trisaccharide ♦ and UDP-tetrasaccharide ▲. Substrate: UDP-Glc □.

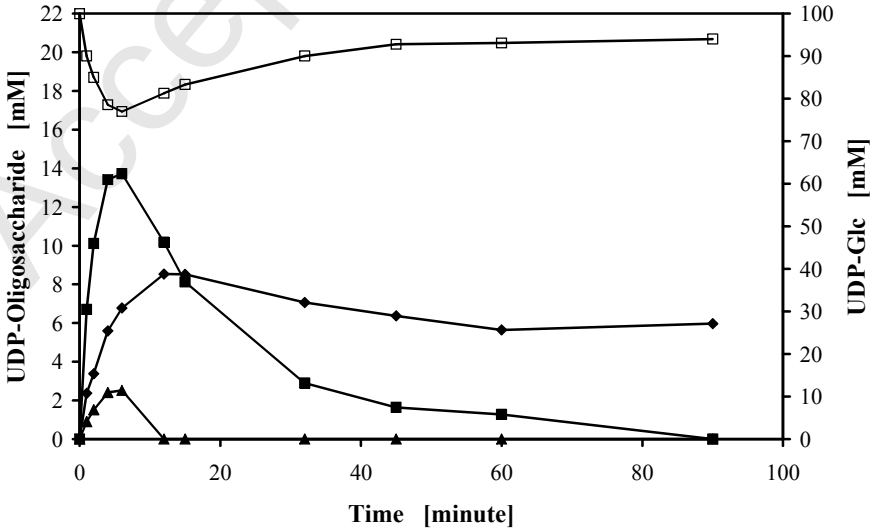


Fig. 3 B. Formation of UDP-activated oligosaccharides at 30 °C with β-galactosidase from *B. circulans* under thermal heating (TH). Products: UDP-disaccharide ■, UDP-trisaccharide ♦ and UDP-tetrasaccharide ▲. Substrate: UDP-Glc □.

The performance of synthesis under MWI has clear advantages and results in a constant product composition over the reaction time. Especially the isolation of the UDP-activated tetrasaccharide is possible with synthesis under MWI. We conclude that MWI improves conditions to produce nucleotide activated oligosaccharides with higher chain length in the transglycosylation reactions by β-galactosidase.

Stable product formation was also obtained for the formation of GAOS (Scheme 1b) under MWI when compared to TH which is also attributed to the suppression of competitive product hydrolysis (see supporting information Fig. S6A and S6B)

3.3. Influence of MWI on the activity of β-galactosidase

The observed short term "activation effect" of MWI on the hydrolytic activity of β-galactosidase cannot explain the stable product formation and obvious suppressed product hydrolysis. We therefore investigated the long term influence of MWI on the hydrolytic reaction of β-galactosidase with *p*NPG as substrate.

The hydrolytic activity of β-galactosidase under reaction conditions identical to those chosen for the synthetic reactions was monitored. With this setup it is possible to determine changes in enzyme activity and address them either to thermal heating or MWI effects. The reactions were carried out in buffers used for transglycosylation reactions at 30 °C with TH or MWI (~20 Watt), respectively.

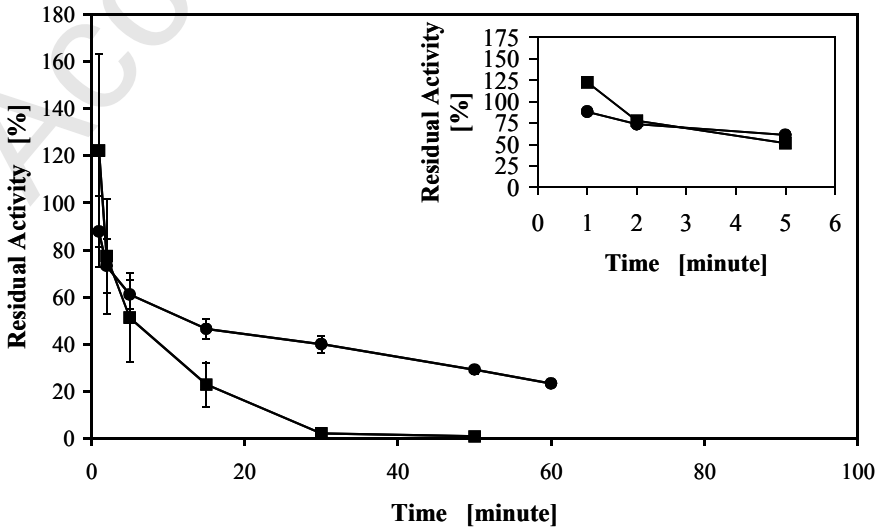


Fig. 4. Hydrolytic activity of β-galactosidase from *B. circulans* at 30 °C under thermal heating (TH) ● and ~20 Watt microwave irradiation (MWI) ■. 100 % residual activity refers to the activity without temperature/MWI pre-incubation. Box: Zoom on the first 5 minutes of residual activity.

The hydrolytic activity of the mesophilic β-galactosidase at 30 °C decreased more rapidly under MWI compared to TH (Fig. 4). Microwave irradiation has a strong influence on the long term activity of the free enzyme. Enzyme activity is almost completely lost after 30 minutes incubation at 20 Watt microwave irradiation, whereas ca. 20 % residual activity remains after 60 minutes under TH. We concluded from these experiments that the rapid loss of hydrolytic activity leads to an increased product stability when free β-galactosidase from *Bacillus circulans* is incubated under MWI at longer incubation time.

This effect depends on the MWI intensity as depicted in Fig. 5: With lower MWI intensities hydrolytic enzyme activity decreases with a slower rate. As a consequence, lower product yields are obtained at a lower MWI irradiation intensity (9 Watt instead of 20 Watt) as demonstrated for the formation of UDP-disaccharide (Fig. 6 and supporting information Fig S5). We conclude that a stable product yield can be tuned by the power of MWI irradiation over incubation time where the enzyme is no longer active to hydrolyse the formed products.

However, reaching slightly higher product yields in relative short incubation times as depicted in Fig. 2 cannot be explained by less product hydrolysis. It can be assumed that the performance of the hydrolytic activity is directly related to the transglycosylation activity of β-galactosidase as lactose hydrolysis is a prerequisite for the transfer of galactose onto an acceptor substrate. A closer look at the first five minutes of incubation reveals an activation effect by MWI on the enzyme hydrolysis reaction which is not observed under TH (insert graphic in Fig. 4). The activity measured under MWI (at ~20 Watt) after one minute is increased (>120 % residual activity) compared to incubation with TH (<100 % residual activity). Moreover, this activation effect can be modulated by MWI intensity as depicted in Fig. 5 (see insert). These results are consistent with the *p*NP-Gal hydrolysis experiment described above (Fig. 2) and suggest that activation of the hydrolytic reaction at the beginning of the incubation with MWI could lead to a higher transfer rate. This finally leads to similar product yields when compared to TH, although enzyme inactivation proceeds more rapidly under MWI. To the best of our knowledge, enzymatic reactions under varying irradiation intensities but identical temperatures have not been studied so far. The variation of the irradiation intensities can be used to adjust the course of a reaction and the formation of single products.

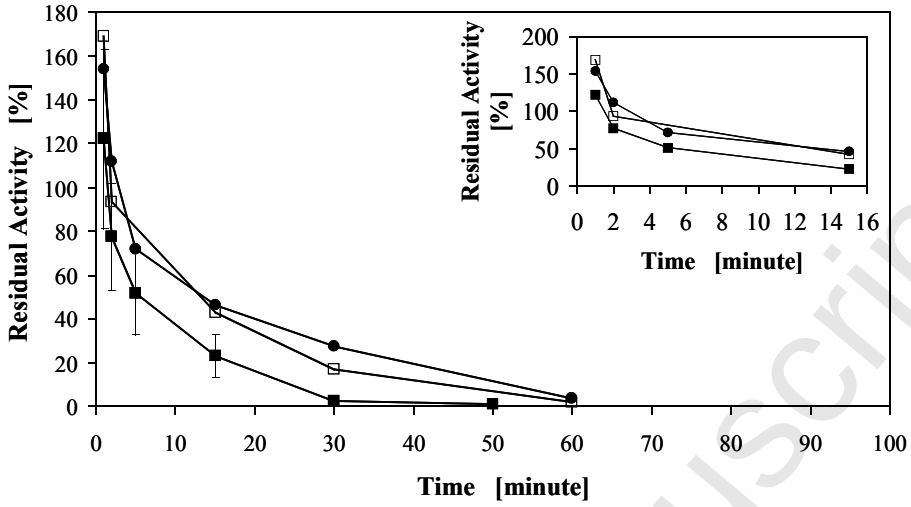


Fig. 5. Hydrolytic activity of β-galactosidase from *B. circulans* at 30 °C and ~11 Watt ●, ~16 Watt □ and ~20 Watt ■ microwave irradiation (MWI). 100 % residual activity refers to the activity without temperature/MWI pre-incubation. Box: Zoom on the first 15 minutes of residual activity.

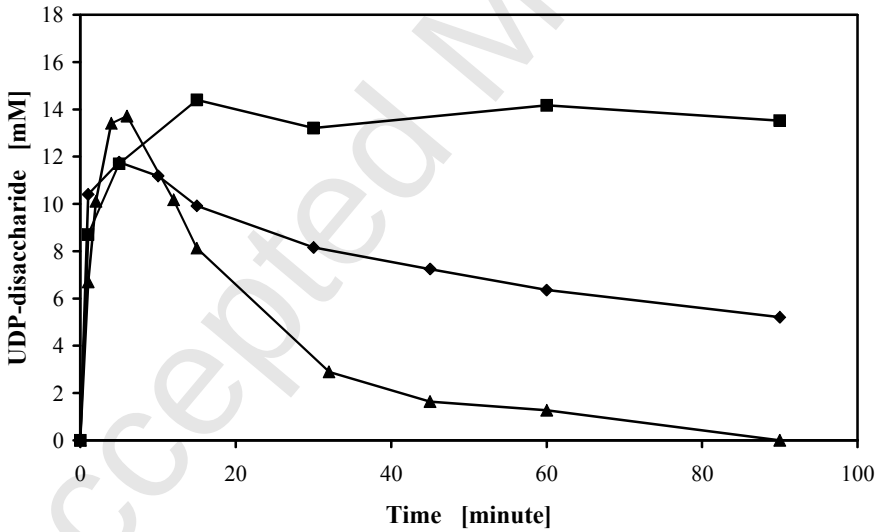


Fig. 6. Comparison of UDP-disaccharide synthesis with β-galactosidase from *B. circulans* and varying irradiation intensities at 30 °C, 500 mM lactose, 100 mM UDP-Glc and ~20 Watt ■, ~9 Watt ♦ microwave irradiation (MWI) and ▲ thermal heating (TH).

Recent examples of relevant literature report as well on increased reaction rates under MWI within short-term experiments with glycosidases [19]. Nogueira et al. reported a microwave effect on enzymatic transesterification reactions [27]. They found an increased activity in the presence of MWI, but also a significant effect of the reaction time in reducing the catalytic activity which they interpreted as enzyme deactivation due to microwave exposure**.**

A drop in enzyme activity was found for a β-galactosidase from *Kluyveromyces lactis* when exposed to 12 Watt at 40 °C over 15 h [20]. MWI here seemed to only affect the free enzyme, while the immobilized *Kluyveromyces* enzyme remained fully active.

3.4. Isolation of the nucleotide sugar fraction and mass spectrometry

Isolation of the nucleotide sugar fraction was accomplished by anion exchange chromatography and subsequent gel filtration for reaction mixtures with UDP-Glc and UDP-GlcNAc, respectively, as acceptor substrates. The purity of the obtained product fractions containing UDP-activated oligosaccharides was analysed by CE-UV using NH_4OAc buffer (Fig. 7A and Fig. 8A). With CE-UV only the UV-active substances, the homologous series of UDP-tetra-, UDP-tri-, UDP-disaccharide, UDP-Glc and UMP and their *N*-acetylated counterparts are visible. The separation proceeds according to size, that is with increasing number of sugar units attached. As counterelectroosmotic analysis at high EOF conditions is used, the largest substances are detected first (Fig. 7A and Fig. 8A).

In order to unambiguously identify the signals present in CE-UV, to also detect non-UV active compounds and enhance the sensitivity, CE-MS analysis was accomplished (Fig. 7B and Fig. 8B). The borate buffer originally used for the CE-UV method (see supporting information Fig. S3) had to be replaced by a CE-MS-compatible buffer containing NH_4OAc at similar pH. The CE-UV separation profile could be preserved and baseline resolution of major analytes could be achieved with this volatile buffer system. Due to the high mass resolution of the qToF-system identification of the analytes was possible without using standard substances. The results of the CE-MS-identification of the nucleotides and sugar-phosphates are given in Table 1 and Fig. 7B and Fig. 8B.

The advantage of the CE-MS method is first of all the possibility to detect also non-UV-active compounds in the reaction mixture such as the sugar phosphates (Glc-phosphate, disaccharide phosphate, trisaccharide phosphate). In addition the sensitivity is greatly enhanced, so the UDP-tetrasaccharide could clearly be identified migrating in front of the UDP-trisaccharide. It is likely, that the separation selectivity changes are due to the change of the buffer system and the coupling to a sheath liquid interface [28], clearly visible for the presence of multiple signals for isobaric compounds: UDP-disaccharide derived from UDP-Glc gives rise to a partially separated triple peak (Fig. 7B), indicating three different isomers. Those are expected to be Gal(β1-4)Glcα1-UDP, Gal(β1-6)Glcα1-UDP and also Gal(β1-3)Glcα1-UDP, as products with UDP-Glc as acceptor substrate. Multiple signals are also visible for the higher

homologues, though the resolution of isobaric regioisomers is lower due to shorter migration times.

Also for the *N*-acetylated counterparts, multiple signals are present: UDP-disaccharide derived from UDP-GlcNAc and the corresponding trisaccharide show double signals in CE-MS (Fig. 8B), indicating two regioisomers. These can be ascribed to Gal(β1-4)GlcNAcαUDP (UDP-LacNAc) and to a lower extent for the Gal(β1-6)GlcNAcαUDP isomer corresponding to the specificity of the enzyme derived from hydrolysis experiments (see supporting information on section 1.2.). The β1-3 regioisomer is not formed, probably because of sterical hindrance through the *N*-acetyl group of UDP-GlcNAc.

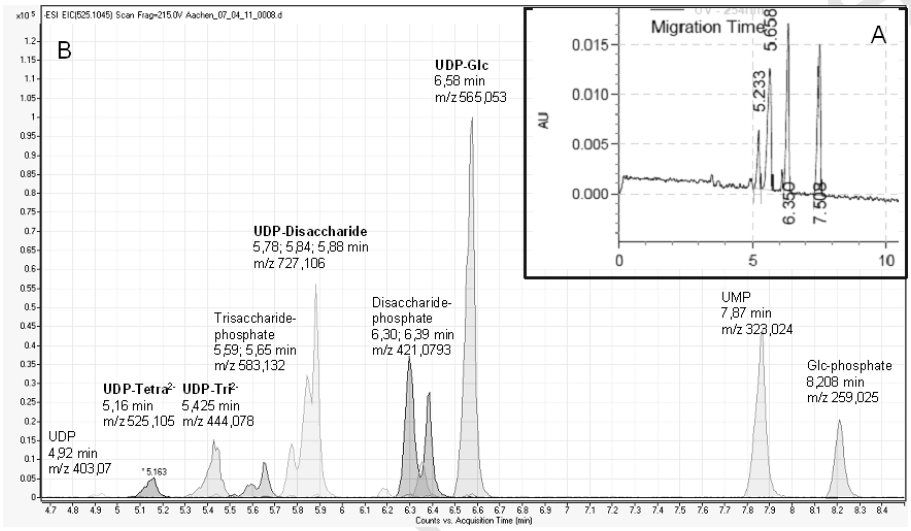


Fig. 7. Electropherogram of isolated nucleotide sugar fraction. UDP-Glc was used as acceptor substrate and lactose as donor substrate to generate nucleotide activated oligosaccharides. (A) CE-UV trace using 50 mM NH_4OAc buffer, pH 9.2. Migration times: UMP (7.5 min), UDP-Glc (6.3 min), UDP-disaccharide (5.6 min), UDP-trisaccharide (5.2) and UDP-tetrasaccharide (trace). (B) CE-MS (negative-ion-mode) extracted ion electropherograms of the same sample. CE-MS conditions see text.

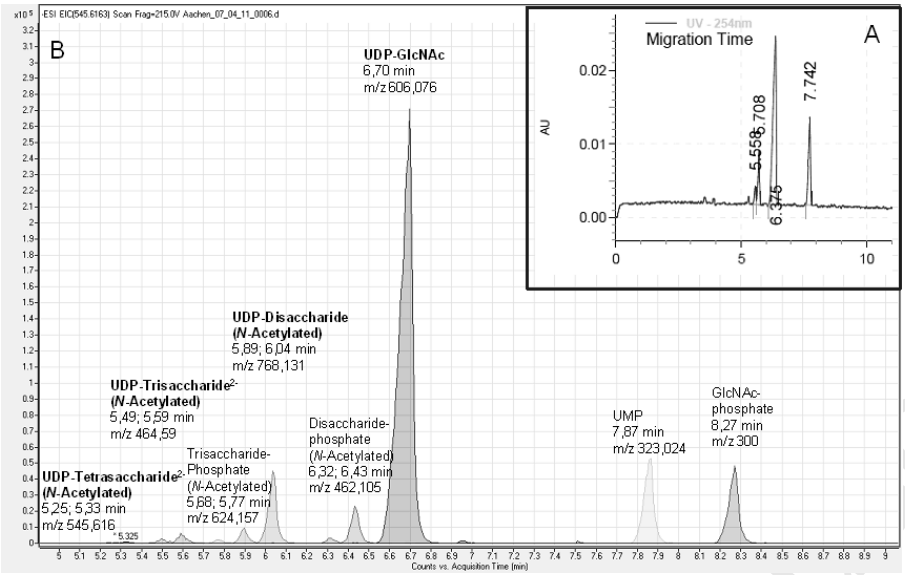


Fig. 8. Electropherogram of isolated nucleotide sugar fraction. UDP-GlcNAc was used as acceptor substrate and lactose as donor substrate to generate nucleotide activated oligosaccharides. (A) CE-UV electropherogram 50 mM NH_4OAc buffer, pH 9.2. Migration times: UMP (7.7 min), UDP-GlcNAc (6.3 min), UDP-disaccharide (5.7 min), UDP-trisaccharide (5.5 min) and UDP-tetrasaccharide (trace). (B) CE-MS (negative-ion-mode) extracted ion electropherograms of the same sample. CE-MS conditions see text.

Tab. 1. Results for the CE-MS analysis of isolated nucleotide sugar fractions where UDP-Glc(NAc) was used as acceptor and lactose as donor substrates to generate nucleotide activated oligosaccharides.

Identified analyte / charge of most abundant charge state	Theoretical mass *m/z*	Observed mass *m/z*	Mass difference (ppm)
UDP / 1-	402.9949	402.9975	6.5
UMP / 1-	323.0286	323.0272	4.3
UDP-Glc / 1-	565.0477	565.0527	8.8
Galβ3,4,6-Glc-UDP / 1-	727.1006	727.1056	6.9
UDP-tri / 2-	444.0731	444.0777	10.4
UDP-tetra / 2-	525.0995	525.1046	9.7
UDP-GlcNAc / 1-	606.0743	606.0785	6.9
Galβ4,6GlcNAc-UDP / 1-	768.1271	768.1304	4.3
UDP-triNAc / 2-	464.5863	464.5902	8.4
UDP-tetraNAc / 2-	545.6127	545.6165	7.0
Glc-phosphate / 1-	259.0224	259.0254	11.6
Disaccharide-phosphate / 1-	421.0753	421.0793	9.5
Trisaccharide-phosphate / 1-	583.1281	583.1325	7.5
Trisaccharide (NAc)-phosphate / 1-	624.1546	624.1572	4.2
Disaccharide (NAc)-phosphate / 1-	462.1018	462.1046	6.1
GlcNAc-phosphate / 1-	300.049	300.0515	8.3

The regioselectivity for β1-4- but not β1-3-glycosidic linkages with UDP-GlcNAc as acceptor is consistent with our previous results [8] where we analysed the regioisomers of the UDP-oligosaccharides synthesised with commercial β-galactosidase from *Bacillus circulans* extensively by NMR. Here, by direct mass analysis out of the reaction mixture and the analysis of the enzyme's regioselectivity by specific hydrolysis of disaccharides with defined glycosidic bonds the product structure can easily be proofed avoiding laborious isolation of single substances.

4. Conclusion

In the present paper we proved the principle of enzyme activation by MWI. However, the activation of the biocatalyst seems to be restricted to the very early period of the reaction (1-3 minutes) demonstrated by *p*NPG-hydrolysis experiments under MWI. This can also be advantageous for transglycosylation reactions to accelerate product formation. Therefore, due to the MWI activation effect maximal product yields can be obtained in a shorter time. In contrast, our long-term experiments show that in the following time period of the reaction (60 minutes) the activity of β-galactosidase decreases rapidly under MWI. The loss of activity seems to be responsible for the minimized hydrolysis of the products under MWI. Hydrolysis of transglycosylation products can thus be prevented by inactivation of the enzyme with MWI. Moreover, we established a new synthetic strategy for the production of UDP-activated oligosaccharides by transglycosylation reactions with commercial β-galactosidase under MWI. The activation of the enzyme in the beginning of the reaction in combination with the following inactivation of the enzyme at longer exposure times was exploited for the synthesis of long-chain UDP-saccharides like UDP-tetrasaccharide. These are not accessible by previously applied methods due to the fast hydrolysis of the tetrasaccharide by β-galactosidase. The identity of the products was assigned by CE-MS. The CE-MS method allows a separation of the reaction products and identification of the compounds by exact mass, directly out of a mixed sample which is impossible with HPLC methods. The CE method has clear advantages for the analysis of nucleotides over HPLC. Most important, ion-pair reagents can be avoided, which means a high degree of freedom to combine CE-devices to mass spectrometers.

We conclude that enzyme activation and deactivation by MWI during in the course of the enzymatic reaction could be exploited for the synthesis of other condensation products using hydrolases. Work is in progress to exploit this strategy for the synthesis of a range of glycoconjugates with glycosidases in our group.

Acknowledgement

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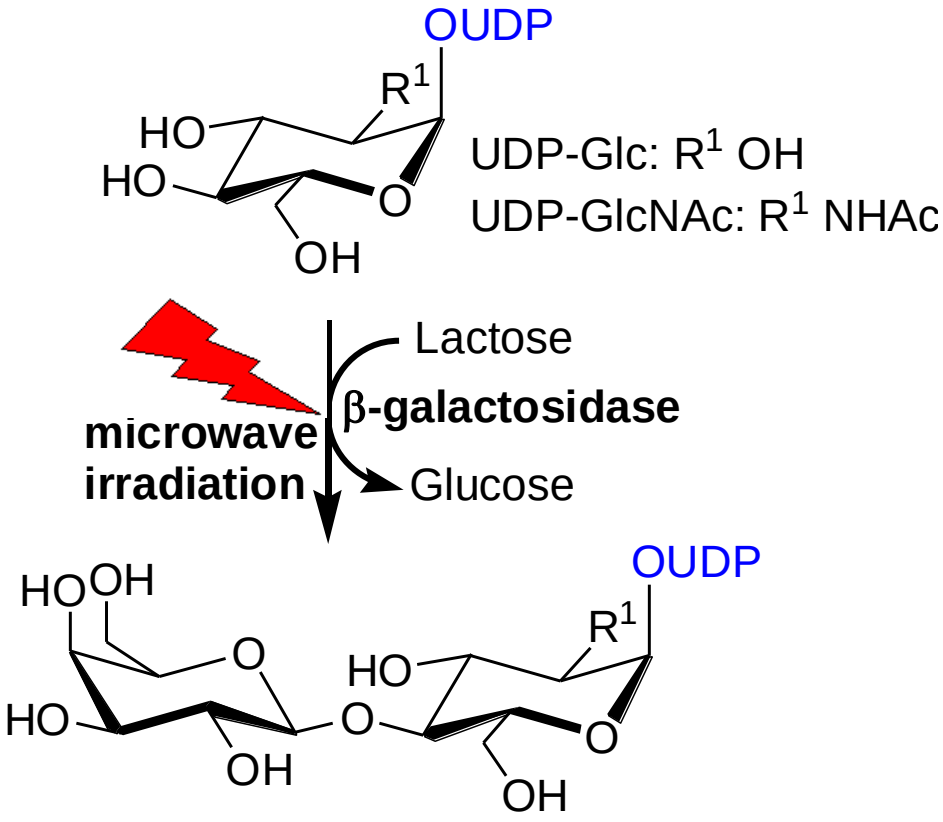
*Graphical Abstract



Highlights

> Enzymatic synthesis under microwave irradiation (MWI) led to stable product yields > Product hydrolysis was significantly reduced under MWI > Oligosaccharide chain lengths up to the tetrasaccharide were achieved