Enzymatic Synthesis of Alkyl Glucosides by β-Glucosidases in a 2-in-1 Deep Eutectic Solvent System

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DOI: 10.1002/cite.202100150

Supporting Information available online

Dedicated to Prof. Dr. Thomas Hirth on the occasion of his 60th birthday

Alkyl glycosides are biodegradable surfactants with excellent physico-chemical properties. Although their wide-range application is considered ecofriendly, their synthesis is not due to the need for toxic organic solvents and corrosive acids as catalysts. Moreover, chemical synthesis results in complex mixtures rather than defined compounds. To overcome both disadvantages as well as the limited solubility of sugars in organic solvents, a highly selective enzymatic synthesis was set up with a β-glucosidase condensing D-glucose and different fatty alcohols in a deep eutectic solvent.

Keywords: Deep eutectic solvents, β-Glucosidase, Glycolipid, Renewables, Tailor-made biosurfactants

Received: July 30, 2021; *revised:* October 04, 2021; *accepted:* November 03, 2021

1 Introduction

Surfactants are amphiphilic and therefore surface-active molecules applied in numerous products of daily life with washing and cleaning being certainly the most prominent field. Further applications include textile manufacturing, cosmetics, emulsifiers in food industry, but also flotation of ores, the production of paints and coatings, foaming agents, in the pharmaceutical industry, and agriculture [1, 2].

Alkyl glycosides (AGs) are non-ionic surfactants containing a mono- or polysaccharide as hydrophilic and a fatty alcohol or fatty acid as hydrophobic moiety, which can both be obtained from renewable resources. In conjunction with ecological compatibility and full biodegradability as well as their excellent physico-chemical properties, AGs are established high-performance components of detergents and cosmetics and have also been suggested as components of water-based lubricants [3–6]. Exact properties of the respective AG depend mainly on the chemical nature of the alkyl chain (length, degree of unsaturation and branching), degree of alkylation and the chemical link between the saccharide and alkyl chain [6–11].

AGs are industrially produced chemically by Fischer glycosylation with good yields at low price on large scale. However, the process employs high temperatures, toxic organic solvents such as trimethylsilyl chloride and corrosive acid catalysts, yielding in compound mixtures of α/β-anomers, pyranoside/furanoside isomers and saccharide polymers of different length resulting in very diverse properties [11–14]. Further limitations result from the poor solubility of saccharides in organic solvents [6, 15].

In accordance with several principles of green chemistry [16] that are inherent to biocatalysis [17], such as waste prevention, reduction of both toxicity of chemicals and use of derivatives or even design for degradation, AGs can be produced enzymatically by using β-glucosidases. The latter naturally hydrolyze glycosidic bonds of polysaccharides. The reaction can be reversed and shifted to condensation in the absence of water, e.g., by using solvents of low water activity. Two mechanisms can be employed, either the reverse hydrolysis pathway (thermodynamically controlled) in which a saccharide and a fatty alcohol are directly connected, or the transglycosylation pathway (kinetically con-

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trolled) in which the saccharide moiety is transferred from an activated saccharide donor to a fatty alcohol [18–20]. The enzymatic reaction operates at mild conditions and results in highly stereo- and regiospecific products. It has been shown that β-glucosidases accept a wide range of aglycons, such as fatty alcohols, hydroxy amino acids and nucleosides enabling the production of a variety of structurally different AGs [21]. Challenges occur from minor solubility of sugars in solvents with low water activity as well as from lacking glucosidase stability when using organic solvents [21].

In early studies, almond β-glucosidase was used to catalyze different alkyl-glucosides in aqueous-organic two phase/biphasic systems or ethoxylated glycoside esters in supersaturated solutions of glucose or *p*-nitrophenyl β-D-xyloside [22–25].

In more recent studies, Bi et al. [26, 27] demonstrated that the addition of ionic liquids as co-solvents to a phosphate buffer-dioxane reaction system could improve reaction rate and yield of salidroside synthesis with β-glucosidase from black plum seed meal. Furthermore, Xu et al. [28] and Uhoraningoga et al. [29] showed that deep eutectic solvents (DES) are suitable co-solvents for β-glucosidase reactions. DESs are a relatively novel class of low-transition-temperature-mixtures consisting of hydrogen-bonding salts and neutral species in the eutectic molar ratio which are liquid at room temperature. Their benign characteristics are like ionic liquids but are in addition easy to prepare from low cost and biodegradable components and have been used in many biocatalytic processes as tuneable co-solvents and solvents of low water activity [30]. Xu et al. [28] compared the performance of *Aspergillus niger* β-glucosidase in different DESs, ionic liquids, organic solvents both as main solvents and co-solvents. They concluded that choline chloride/propylene glycol DES (ChCl:PG) either with 40 % buffer as co-solvent or with 6 vol % water as main solvent performed best, as activity was improved by 225 % (DES as co-solvent) or enzyme stability was retained on a high level over 5 days (DES as main solvent). Similar effects were observed for *Streptomyces griseus* β-glucosidase in 40 vol % choline chloride/glyerol DES-buffer systems [28].

Besides having beneficial effects on stability and activity of enzymes, DESs can also be used as 2-in-1 systems. For glycolipid synthesis with lipases in DES, it was shown that either the fatty acid or the sugar component can be part of the hydrophobic or hydrophilic DES, respectively, but at the same time also be a substrate for the enzymatic reaction [31–36]. Aim of the presented study is to transfer this concept to β-glucosidase reactions. Furthermore, performance of a technical enzyme formulation is compared to purified almond β-glucosidase and further optimized regarding alkyl chain length, fatty alcohol concentration, water content and reaction time.

2 Materials and Methods

2.1 Chemicals

All chemicals, including standards of studied AGs, were purchased from either Carl Roth GmbH & Co. KG (Karlsruhe, Germany) or Sigma Aldrich Chemie GmbH (Taufkirchen, Germany) if not stated otherwise. All solvents were HPLC grade.

2.2 Enzymes

Two different formulations of β-glucosidase enzyme (β-D-glucoside-glucohydrolase; EC 3.2.1.21) were used in this study. Firstly, β-glucosidase from almond was purchased from Sigma-Aldrich as lyophilized powder containing $\geq$ 2 units/mg solid (CAS 9001-22-3; G0395). The second β-glucosidase GC 151 (batch 4902629386) was a liquid technical enzyme formulation of microbial origin produced by Genencor International, B.V. containing 5–10 wt % enzyme with a minimal activity of 5400–6600 U g^{-1}. The formulation was further composed of 54.4–69.6 % water, 25–35 wt % dextrose and 0.4–0.6 wt % potassium sorbate and had a pH of 5.0–6.0.

To ensure comparable reaction conditions, 25 mg almond β-glucosidase was dissolved in 60 µL phosphate-citrate-buffer according to McIlvaine [37]. Briefly, 515 mL of 0.2 M sodium di-hydrogen phosphate was mixed with 485 mL of 0.1 M citric acid resulting in 1 L of buffer with a pH of 5.0.

2.3 Alkyl Glucoside Synthesis in Deep Eutectic Solvent

A choline chloride-glucose DES was used as reaction medium of low water activity for all enzymatic synthesis reactions. Therefore, exicator dried choline chloride and glucose were mixed in a molar ratio of 2:1, heated to 95 °C and stirred until it became a clear and homogenous liquid. In addition to serve as a solvent, the contained glucose acted as substrate for AG synthesis forming thus, a 2-in-1 concept (Fig. 1).

The substrate range experiments were conducted in a total volume of 1.25 mL. Therefore, 1.1 mmol of different fatty alcohols (1-butanol, 1-hexanol, 1-octanol, and 1-decanol) were added to the choline chloride-glucose DES in 5 mL reaction tubes (Eppendorf AG, Hamburg, Germany). To start the reaction, either 60 µL of almond β-glucosidase solved in citrate-phosphate buffer or 60–240 µL of GC 151 were added as indicated in the results section. As negative control, 240 µL of citrate-phosphate buffer without enzyme was used. The reaction was conducted at 50 °C for 72 h in a rotator with a vortex mixer (program U2) from NeoLab (Heidelberg, Germany) at 90 rpm. The reactions were stopped by adding twice 2 mL ethyl acetate

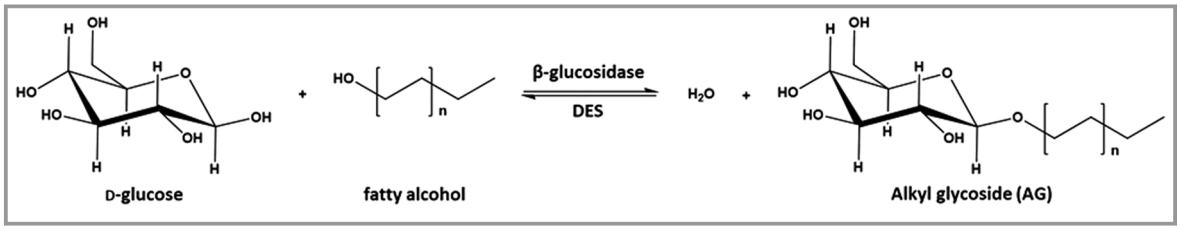

Figure 1. Tailor-made alkyl glycosides (AGs) are prepared from D-glucose and fatty alcohols with n = 2, 4, 6 and 8, using β-glucosidases. Reactions were performed in a deep eutectic solvent (DES) consisting of choline chloride and D-glucose, the latter simultaneously serving as substrate and solvent (2-in-1 reaction concept).

and vigorous mixing. The upper phases containing AGs were collected, unified, and concentrated in a vacuum concentrator. Qualitative analysis was performed by thin-layer chromatography (TLC).

Experiments elucidating optimal fatty alcohol concentration were conducted in a total volume of 3.5 mL in 5-mL reaction tubes. Different amounts of 1-butanol, 1-hexanol or 1-octanol were added to the choline chloride-glucose DES: 0.2 mmol, 0.4 mmol, 1 mmol, 2 mmol, 4 mmol or 6 mmol. The reaction was started by adding 960 µL of GC 151 and was conducted in triplicates at 50 °C for 72 h in a rotator with a vortex mixer (program U2) at 90 rpm. The reactions were stopped by transferring the reaction mixture to a 15-mL reaction tube, adding 800 µL water and 8 mL ethyl acetate. After mixing for 1 min by vortexing, the upper phase was analyzed via HPLC.

Experiments to study the influence of water content were performed in a total volume of 3.5 mL in 5-mL reaction tubes. Water was added to the DES to yield water contents of 2.5, 5, 7.5 or 10 wt %. Subsequently, 4 mmol of fatty alcohol and 960 µL of GC 151 were added. The reaction was conducted at 50 °C either for 96 h (butanol or hexanol) or 192 h (octanol) in a rotator with a vortex mixer (program U2) at 90 rpm. The reaction was stopped and analyzed as described for the fatty alcohol concentration experiments.

Time course experiments lasted over 8 days (192 h) with samples taken every 24 h. For each fatty alcohol (1- butanol, 1-hexanol and 1-octanol), nine identical reaction mixtures of 3.5 mL total volume were prepared consisting of 4 mmol fatty alcohol, 7.5 wt % water and 960 µL GC 151. Reactions were started by adding the enzyme and were conducted at 50 °C in a rotator with a vortex mixer (program U2) at 90 rpm. For each sampling point (0 h, 24 h, 48 h, …, 192 h) the reaction in one of the tubes was stopped by adding 800 µL water and 8 mL ethyl acetate. Reactions were performed in duplicates. In addition, a negative control containing citrate-phosphate buffer instead of enzyme was conducted. All samples were analyzed by HPLC.

2.4 Analytical Methods

2.4.1 Thin-Layer Chromatography

For a rapid qualitative control of AG synthesis, thin-layer chromatography was performed. The samples were spotted onto a silica plate (ALUGRAM® SIL G 0,2 mm, Macherey-Nagel). A standard of the respective AG (butyl-β-glucopyranoside, hexyl-β-glucopyranoside or octyl-β-glucopyranoside, as well as a JBR 425 rhamnolipid standard from Jeneil (Saukville, Wisconsin, USA), was always included as a reference. In each case, 5 µL of sample and 0.75 µL of standard (10 mg per 1 mL ethyl acetate) were applied. The TLC development chamber was filled with the eluent (chloroform: methanol: acetic acid 6.5:1.5:2 (v/v)) 10 min in advance to ensure that the chamber was saturated with the eluent.

After developing the TLC plate for about 30 min, the plate was briefly immersed in thymol staining agent (2 g thymol in 190 mL ethanol (96 %) and 10 mL sulfuric acid (96 %)). Stained AGs were visualized by heating the plate to 200 °C for one minute using a hot air dryer.

2.4.2 Flash Chromatography

Since unreacted fatty alcohol, as well as small amounts of glucose, were always present in extracted samples, the AG-containing phase was further fractionated by preparative flash chromatography before applying them to NMR analysis.

Extracts were first concentrated to about 2 mL in a rotary evaporator. The column used for flash chromatography was FlashPure Ecoflex Silica (50 µm; 25 g) from Büchi. The eluent consisted of chloroform and methanol, working with a gradient in which the proportion of methanol was increased over time: starting at 0 % methanol for the first 3.5 min, methanol concentration was increased to 8 % within 1.9 min. Subsequently, it was further increased to 10 % within 11.1 min, followed by an increase to 100 % within 1.5 min. The flow rate was 28 mL min^{-1}.

The individual fractions were automatically collected into 15-mL centrifuge tubes during this process. Thin-layer chromatography was performed to check for clean fractionation. Fractions containing AGs were combined.

2.4.3 High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) was performed for quantitative analysis of AG syntheses. AGs were detected by an evaporative light scattering detector (ELSD). The column used was a Luna® Omega Sugar column (250×4.6 mm, 3 µm, 100 Å) from Phenomenex®. The column temperature was set to 35 °C, and the detector temperature was 40 °C. Acetonitrile and water (ddH_2O) were used as eluent, and a gradient was used: Starting with 95 % acetonitrile for the first 1.5 min, acetonitrile concentration was reduced to 75 % within 1 min and was hold for 1 min. Subsequently, acetonitrile concentration was brought again to 95 %, which was hold until the end of the run. The flow rate was 1 mL min^{-1}.

Because the AG concentrations in the extractions were sometimes very low, measurements were performed with 1 µL injected sample volume and a detector signal amplification of gain 2, as well as with 5 µL injection volume and an amplification of gain 16.

2.4.4 NMR and Mass Spectrometry

For nuclear magnetic resonance (^{1}H NMR) spectroscopy, in DMSO-d6 (CD_2Cl_2 purchased from Eurisotop, Saarbrücken, Germany) on an Avance 400 NMR instrument (Bruker Biospin GmbH, Rheinstetten, Germany), with 300 MHz for ^{1}H. The chemical shifts are expressed in δ (ppm) versus tetramethylsilane (TMS) = 0. These results were processed with MestReNova 14.2.0 (Mestrelab Research S.L., Santiago de Compostela, Spain).

13-NMR and 2D-NMR in $CHCl_3$/MeOD 70:30 (^{1}H-^{13}C heteronuclear single-quantum correlation (HSQC) spectroscopy and ^{1}H-^{13}C heteronuclear multiple-bond correlation (HMBC) spectroscopy were performed on a Bruker AVANCE III 600 MHz spectrometer (Bruker BioSpin, Rheinstetten, Germany) equipped with a TCI cryoprobe at a temperature of 27 °C. Spectra were processed and analyzed using Topspin 4.0.1 (Bruker BioSpin) and chemical shifts were referenced ^{13}C resonance of tetramethylsilane (TMS).

The mass spectrometry (MS) for mass identifications was performed with electrospray ionization (ESI) on a quadrupole Q Exactive Plus (ThermoFisher Scientific GmbH, Kandel, Germany) and recorded in positive mode, raw spectrometric data was treated using MestReNova Suite 2020 [version 14.2.0] (Mestrelab Research S.L., Santiago de Compostela, Spain).

3 Results

3.1 Substrate Screening

The alkyl glucoside synthesis from C4 to C10 fatty alcohols and glucose in a 2-in-1 DES using a β-glucosidase isolated from almonds was proven successful as shows the TLC in Fig. 2A. Indeed, the spots obtained (after TLC dying) with retention factor (Rf) values of 0.42, 0.45, 0.47 and 0.49 correspond to butyl, hexyl, octyl and decyl glucosides, which are like the Rfs obtained using the AG standards. Besides, only a single spot of unreacted glucose with a Rf value of 0.11 was observed. The crescent-shaped spots below the elution front corresponds to the various unreacted fatty alcohols. Sensible differences between the Rf values of the enzymatically synthesized glycolipids and the standards can

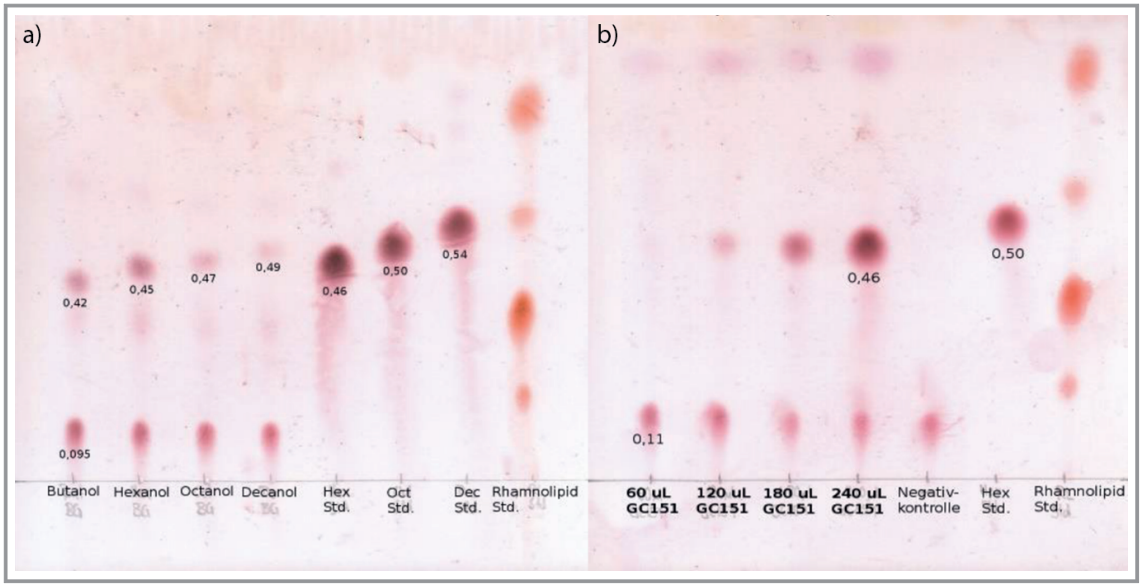

Figure 2. Alkyl glucoside synthesis with A) β-glucosidase isolated from almonds, 25 mg enzyme in 60 µL citrate buffer, 1.1 mmol of each fatty alcohol (C4 to C10); B) β-glucosidase liquid formulation GC 151 and 1-butanol, amount of enzyme used varied from 60 to 240 µL. For both conditions: 48 h reaction at 50 °C, in a glucose-based DES containing 7.5 wt % water. Elution of TLC with $CHCl_3$, methanol, acetic acid (65:15:2 v/v/v), thymol staining.

be attributed to the additional presence of glucose (Rf ≈ 0.1), which dissolves to a minimal extent in the ethyl acetate and logically was extracted. This changes the running behavior of the glycolipids, which explains the slightly deviated Rf values.

The commercially available enzyme that was used, to prove the principle, is however very costly (~60 € kU^{-1}), thus a less expensive alternative was needed to make the process relevant in the aim of an eventual scale-up. The β-glucosidase GC 151, a buffered formulation, was introduced as a substitute to carry the production of the targeted compounds. Fig. 2B demonstrates this approach, as it repeats the synthesis of butyl glucoside. While varying the amount of technical enzyme used, TLC displaying spots with similar Rfs were obtained. In Fig. 3 the rest of the expected AGs based on this principle are displayed. As the amount of technical enzyme increased, the observed intensity of the spots also increased, thus 240 µL of technical enzyme was used in the following experiments.

Nonetheless, as it can be seen in the result of the octyl glucoside synthesis (Fig. 3B), the product spots obtained were getting weaker compared to the experiment using butanol or hexanol (Fig. 3A) as substrates. This tendency becomes even clearer when considering the result of the synthesis using decanol as substrate (Fig. 3C). Qualitatively, this experiment shows that with an increasing length of the alkyl chain attached to the primary alcohol, the performance of the β-glucosidase diminishes to the point that barely any visible decyl glucoside stain was formed. Based on these results, further experiments using decanol as a substrate were dispensed. In the further course, work was focused on the use of butanol, hexanol and octanol as substrate for the biocatalyzed reaction.

Confirmation of the structures using mass spectrometry and NMR analysis was thereafter issued.

3.2 Structure Elucidation using Spectroscopic and Spectrometric Methods

For the mass spectrometric analyses, ESI-Q-TOF mass spectra were recorded in positive mode. The combination of the latter to not only ^{1}H-and ^{13}C-NMR analysis but also to ^{1}H-^{1}H COSY, ^{1}H-^{13}C HMBC and ^{1}H-^{13}C HSQC experiments enabled a thorough structural elucidation for each of the compounds scrutinized in the present work.

The chemical shifts of butyl-β-D-glucopyranoside (BGP) are given as follows (cf. Fig. S1 in the Supporting Information SI):

^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm), 4.97–4.82 (m, 3H), 4,46 (t, J = 5.8, 5.8 Hz, 1H), 4.09 (d, J = 7.8 Hz, 1H), 3.83–3.59 (m, 2H), 3.49–3.33 (m, 2H), 3.18–2.85 (m, 3H), 1.58–1,42 (m, 2H), 1.44–1.20 (m, 3H), 0.87 (t, J = 7.3, 7.3 Hz, 3H).

^{13}C NMR (600 MHz, $CDCl_3$/MeOD 70:30) δ (ppm), 102.9, 77.2, 76.3, 70.8, 70.5, 70.2, 69.9, 32.6.

Fig. 4 shows the mass spectrum of the purified glycolipid fraction from the synthesis of butyl-β-D-glucopyranoside (BGP) using GC 151. The molecular mass of the corresponding compound (M_{BGP}) is 236.26 g mol^{-1}; $[M_{BGP} + H]^+$ 237.13, $[M_{BGP} + Na]^+$-H_2O 241.14, $[M_{BGP} + NH_4]^+$ 254.16, $[M_{BGP}+H]^+$+CH_3OH 269.14.

Chemical shifts and mass spectra of hexyl-β-D-glucopyranoside (HGP) and octyl-β-D-glucopyranoside (OGP) are listed in the SI (Figs. S2 and S3).

3.3 Quantification of the Produced Alkyl Glucosides

The different AGs were successfully separated and quantified using a newly developed analytical HPLC-ESLD method, which allowed the detection of single peaks

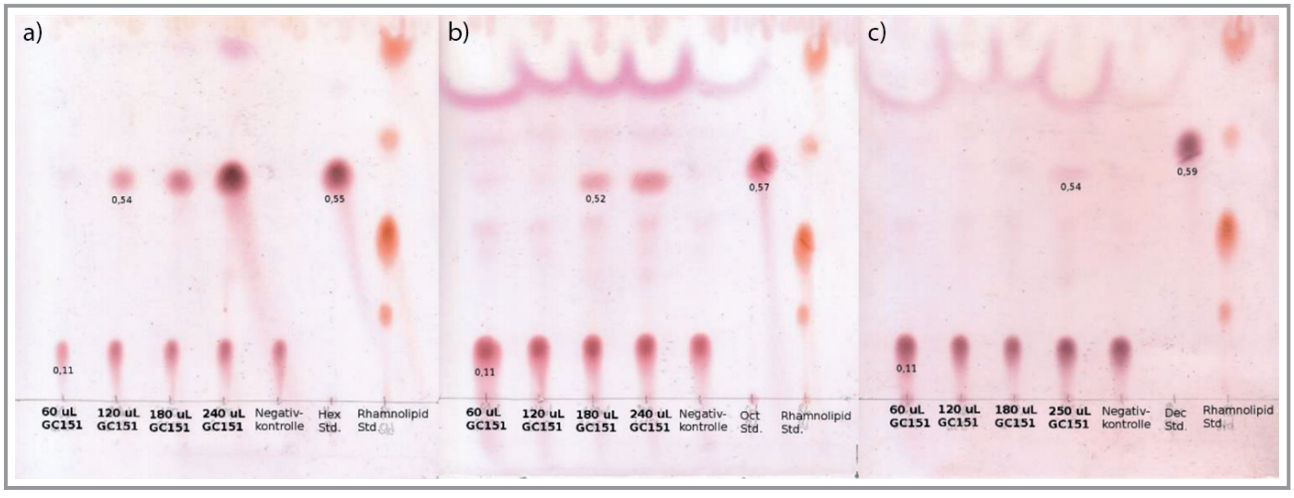

Figure 3. Alkyl glucoside synthesis using β-glucosidase liquid formulation GC 151 with A) 1-hexanol, B) 1-octanol, C) 1-decanol, and as substrates. For all conditions: amount of enzyme used varied from 60 to 240 µL (250 µL max. for decanol only), 48 h reaction at 50 °C, in a glucose-based DES containing 7.5 wt % water. Elution of TLC with $CHCl_3$, methanol, acetic acid (65:15:2 v/v/v), thymol staining.

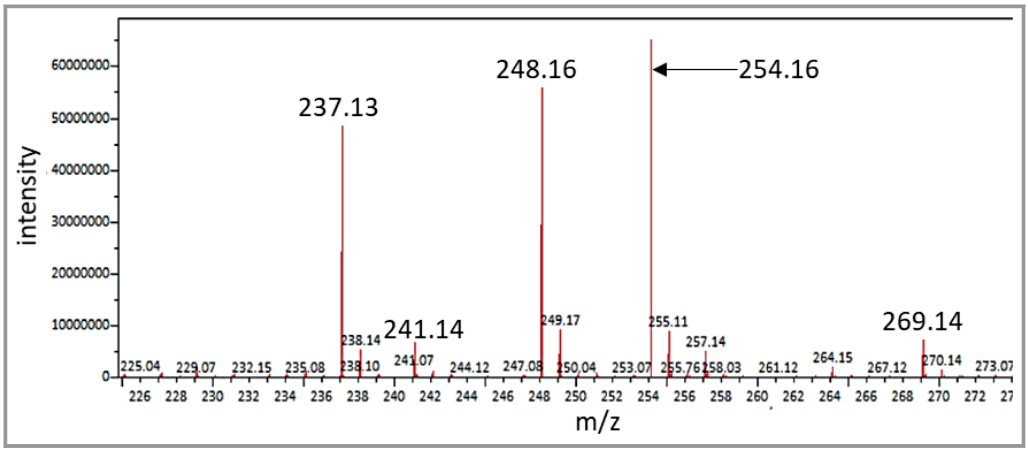

Figure 4. Mass spectrum of the isolated AG fraction from the synthesis using 1-butanol as substrate.

(Fig. S4–S6). It was possible to quantify BGP, HGP and OGP in a range between 0.025 g L^{-1} and 0.3 g L^{-1} with the use of polynomial ranges of calibration as ELSD did not provide a linear response when concentration of the analytes increased (Tab. 1).

3.4 Factors Influencing the Synthesis

Herein, factors impacting the reaction (i.e., time of reaction, substrate concentration and water content in the media) have been investigated to find the optimal parameters for each condition and different AGs. From Fig. 5–7, it can be discerned that the optimal values were, in average, 7.5 wt % water, 2 mmol of fatty alcohol and 6 days of reaction.

3.4.1 Water Content

The results displayed in Fig. 5 are rather unambiguous concerning the water content. Indeed, above 5 wt % water, it seemed that a conducive range of water content was reached for the production of AGs. Thus, an average optimal water content of 7.5 wt % could be determined for BGP and OGP, possibly the production of HGP could perform as efficient up to 10 wt % of water content.

Table 1. Chromatographic and analytical characteristics of produced AGs (BGP/HGP/OGP) using HPLC-ELSD.

Retention time (AGs)[a),b)] [min]	6.88–7.02
Polynomial range of calibration [g L^{-1}]	0.025–0.3
Peak width[a),b)] [min]	0.058–0.075
Baseline noise[a),b)] (n = 3) [mV]	0.19 ± 0.06
Limit of detection[a),b)] (signal/noise = 3) [g L^{-1}]	< 0.013
Limit of quantification[a),b)] (signal/noise = 10) [g L^{-1}]	0.013
BGP calibration (Fig. S4)	
Correlation coefficient (R^2, n = 3)	0.9997
Equation of polynomial calibration	$y = 50879x^2 + 54506x - 411.95$
HGP calibration (Fig. S5)	
Correlation coefficient (R^2, n = 3)	0.9999
Equation of polynomial calibration	$y = 80571x^2 + 35874x - 453.04$
OGP calibration (Fig. S6)	
Correlation coefficient (R^2, n = 3)	0.9964
Equation of polynomial calibration	$y = 40264x^2 + 58529x - 1380.3$

a) Averaged values of BGP/HGP/OGP based on inter-day variance of retention time measured on different days. b) Concentration 0.025–0.3 g L^{-1}.

3.4.2 Fatty Alcohol Concentration

Drastic differences are to be observed in Fig. 6 as 2 mmol of butanol induces a two-fold increase in titer compared to 1 mmol. Amount of BGP increased significantly with increasing concentration of butanol up to 4 mmol. Further increase of butanol concentration led to a decrease of BGP. The amount HGP instead was constant with a hexanol concentration of 0.2–2 mmol with a slight decrease of HGP amount at higher hexanol concentrations. However, no significant correlation between the amount of OGP and the octanol concentration was observed with OGP showing lower yields than the other AGs. This suggests that upon a range of concentration > 2 mmol, there was potentially an inhibition of the bio-

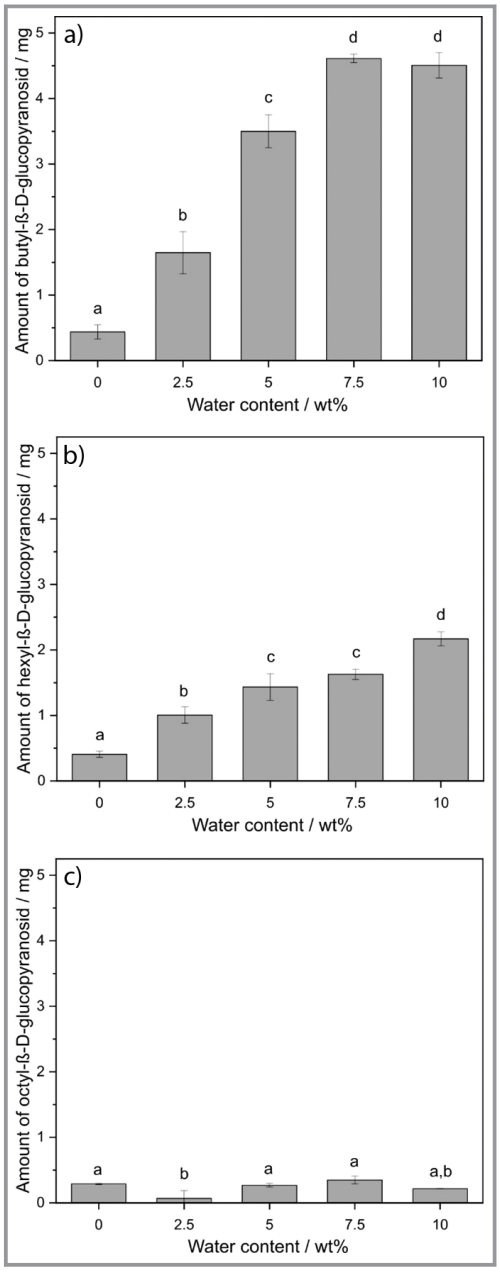

Figure 5. Effect of water content in the media on the β-glucosidase-catalyzed glycosylation reaction between glucose and A) 1-btuanol, B) 1-hexanol, and C) 1-octanol. Error bars are expressed as standard deviations of the mean values (n = 3). a–d show statistically significant differences, at a 0.05-significance level of the mean values obtained from three independent experiments ran under each condition.

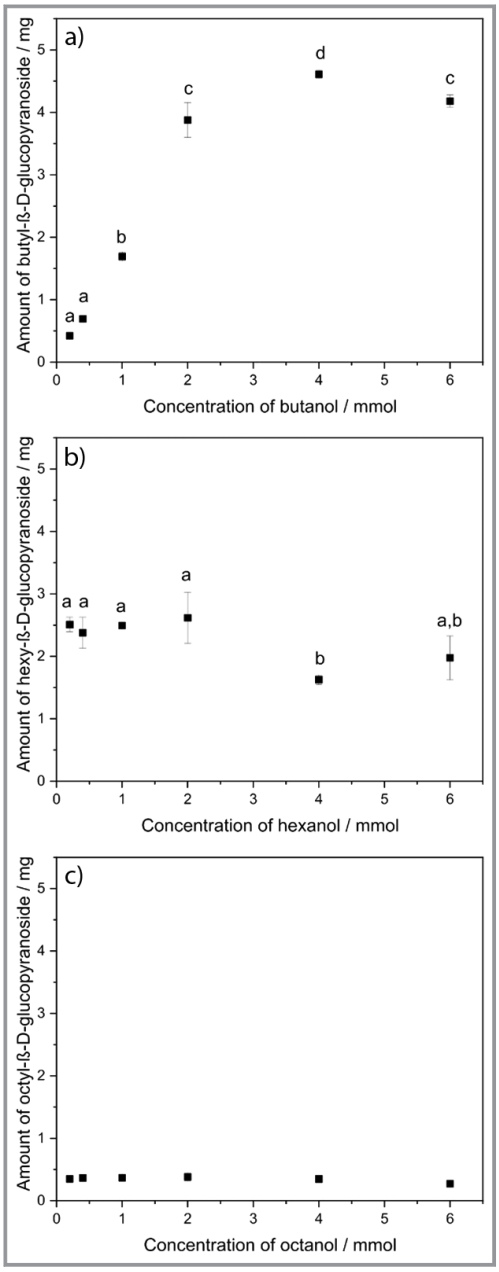

Figure 6. Effect of substrate incrementation in the media on the β-glucosidase-catalyzed glycosylation reaction between glucose and A) 1-btuanol, B) 1-hexanol, and C) 1-octanol. Error bars are expressed as standard deviations of the mean values (n = 3). a–d show statistically significant differences, at a 0.05-significance level of the mean values obtained from three independent experiments ran under each condition.

catalyst due to substrate saturation during the production of HGP and OGP.

3.4.3 Product Formation over Time

To determine the optimal reaction time to produce the maximum amounts of AGs, BGP was chosen as a model product and its concentration over 8 days of reaction. Thus, a saturation of product after 6 days with apparently no significant changes in concentration was reached, even up to 8 days of reaction, as shown in Fig. 7.

Under these optimized conditions using the β-glucosidase formulation GC 151, a mass of 4.03 ± 0.25 mg of product was obtained after 6 days of reaction, which translates to a

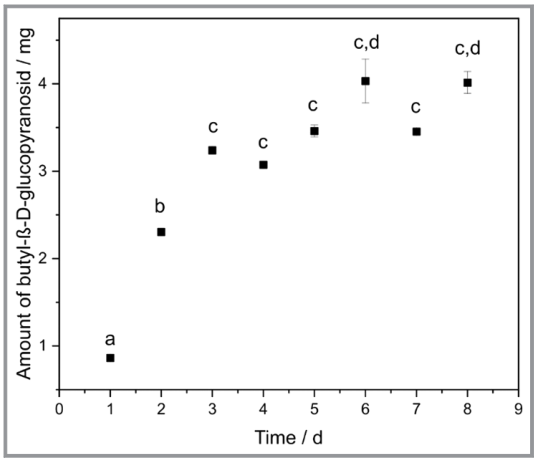

Figure 7. Time course of octyl-β-D-glucopyranoside (OGP) production as model for the production of short chained AGs. a–d show statistically significant differences, at a 0.05-significance level of the mean values obtained from three independent experiments ran under each condition.

specific productivity of 100 µmol $h^{-1}g^{-1}$. Until now, no data has been reported regarding the use of a 2-in-1 DES system for β-glucosidase-catalyzed glycosylation between short chained fatty alcohols and monosaccharides to synthetize such monoglycosylated hexoses.

4 Discussion

In contrast to chemical and microbial synthesis, the enzymatic synthesis of AGs using hydrolases under almost anhydrous conditions offers the possibility of tailor-made production of surfactants for detergent and emulsifier applications [30, 38]. In addition to the selection of inexpensive technical enzymes with high activity and high regioselectivity for synthesis, two major challenges remain: choosing reaction media that not only solubilizes well the sugar- as well as the fatty acid- or fatty alcohol-substrate-components but also retains the activity and stability of enzymes over longer time periods.

Deep eutectic solvents (DES) that are made from inexpensive renewable raw materials [30] are an interesting alternative to organic solvents [25] or ionic liquids [26]. After the successful demonstration of various sugar synthons esterification with different fatty acid building blocks employing lipases in DES [39, 40], whereby the sugar component can act as a solvent component and as a substrate at the same time [33, 35, 36, 40], this study showed that glycosidases can also be used in DES for the synthesis of AGs according to the 2-in-1 concept. In other studies, presented so far, DES had mainly been used as a co-solvent [26–29]. In addition, the use of an inexpensive technical enzyme formulation instead of a highly pure enzyme from almonds was demonstrated for the first time.

A great advantage of an enzymatic synthesis in DES compared to a synthesis in organic solvents is the possibility to optimize the water activity based on the water content. In the presented study, a content of 7.5 wt % water, which is not preferable only for the mass transfer, but also beneficial for maintaining enzyme activity therein and enzyme stability, was found to be optimal. Enzymes require a minimal amount of water to maintain their hydration shell and stay active. This behavior observed is not singular to β-glucosidases, indeed, lipases were also reported to exhibit the need for a minimum amount of water in a similar range of concentration (5–10 wt %) [33, 36, 40]. However, the investigated β-glucosidases operate at relatively low water content compared to other glycosidic enzymes as for example, α-amylases required at least 10–20 % of water as co-solvent to show activity [20]. Otto et al. [24] reported that the reaction rate of almond β-glucosidase was highest with 10 % water content and decreased drastically at lower water contents. However, after immobilizing the enzyme on Eupergit C™, it was active even at only 1 % water content. To overcome solubility limitations of the sugar in aqueous-organic two-phase systems and hence, mass transfer limitations, Yi et al. [23] stabilized the dispersed aqueous phase in the form of permeable microcapsules containing the enzyme that was saturated with glucose. In consequence, microencapsulated β-glucosidase stayed active over longer periods and could be reused in batch experiments after replenishing glucose.

However, the yields achieved therein are relatively low. Thus, next investigations should aim at intensifying the performances of the process in terms of space-time yields and turnover rates. Carrying out this enzymatic synthesis in a microwave reactor can be seriously considered as it was successfully applied to the lipase-catalyzed synthesis of glycolipids in prior reports [35].

In contrast to the lipase-catalyzed synthesis of glycolipids, the preference of the glycosidase reaction is towards shorter alkyl chains. A possible explanation for the observed decrease in glycolipid production with increasing chain length may be steric hindrance. Due to the longer alkyl chain, the similarity with the natural substrate glucose decreases, so that the longer-chained fatty alcohols fit poorly into the active site of the enzyme ending up into productivity decrease. Another possible cause may be correlated to the polarity of the fatty alcohols. Indeed, as the length of the alkyl chain increases, its hydrophobicity increases and its polarity decreases. Thus, the similarity to the strongly polar glucose, the natural product, decreases progressively. Moreover, fatty alcohols with longer chains might be less well distributed in the DES than the hydrophobic butanol, as a limited distribution of fatty acids in hydrophilic DES was reported earlier [33]. However, in contrast to the lipase catalyzed reaction and chemical glycosylation, glucosidases have a very high regioselectivity, so that no complex protection/deprotection strategy is needed to selectively to achieve the desired product. In addition to

increasing the reaction rate and reaching higher product concentrations, it will be of great interest to expand the product range for DES preparation and enzymatic synthesis by using other sugar components and investigate purification strategies to remove unreacted substrates without use of chromatography [4, 21].

In summary, it can be said that there is great potential in using glycosidases for the synthesis of AG surfactants in bio-based inexpensive DES as unconventional reaction media.

Supporting Information

Supporting Information for this article can be found under 10.1002/cite.202100150. This section includes 6 supplemental figures (Figs. S1–S6) to support this research.

The authors gratefully acknowledge the team of Stefan Bräse from the Institute of Organic Chemistry of the KIT (Karlsruhe, Germany) for performing the MS and NMR analysis. Open access funding enabled and organized by Projekt DEAL.

Abbreviations

AG	akyl glycoside
BGP	butyl-β-D-glucopyranoside
DES	deep eutectic solvent
HGP	hexyl-β-D-glucopyranoside
OGP	octyl-β-D-glucopyranoside

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DOI: 10.1002/cite.202100150

Enzymatic Synthesis of Alkyl Glucosides by β-Glucosidases in a 2-in-1 Deep Eutectic Solvent System

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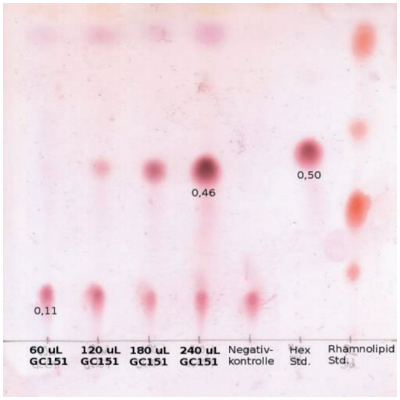

Research Article: The study achieved a highly selective synthesis of alkyl glycosides using a β-glucosidase that allowed condensation between D-glucose and different fatty alcohols in a deep eutectic solvent (DES). Thereby, the reaction system overcomes limitations of low sugar solubility in organic solvents and need for complex protection/deprotection strategies.

Supporting Information available online

