Research Article

Lipase-Mediated Mechanoenzymatic Synthesis of Sugar Esters in Dissolved Unconventional and Neat Reaction Systems

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ABSTRACT: Mechanochemical and biocatalytic approaches in modern research are two major assets to develop greener processes. In the present study, these modular tools of sustainability are pointed toward the production of versatile and daily employed compounds such as surfactants. Toward this aim, glycolipids, a class of nonionic surfactants composed of ubiquitous and primary metabolites such as sugar and fatty acid moieties, represent a promising alternative to petroleum-derived surface-active agents. Therefore, the combination of biocatalysis with mechanochemistry aiming at glycolipid synthesis seemed a logical step that was taken in this study for the first time. The monoacylated model compound glucose-6-O-decanoate was synthesized with the help of a bead mill apparatus using two different unconventional dissolved reaction systems, namely, menthol-based hydrophobic deep eutectic solvents and 2-methyl-2-butanol, thus reaching up to 12% yield in the latter based on the conversion of vinyl decanoate, after only 90 min of reaction. In addition, a neat reaction system using an excess of vinylated fatty ester as an adjuvant allowed a 27 mM/h space-time yield. The overall significant increase in productivities, up to 6 times, compared to standard heating and shaking methods, shows the tremendous potential of mechanoenzymatic synthesis.

KEYWORDS: biocatalysis, solvent-free, transesterification, glycolipid, lipase, biosurfactants
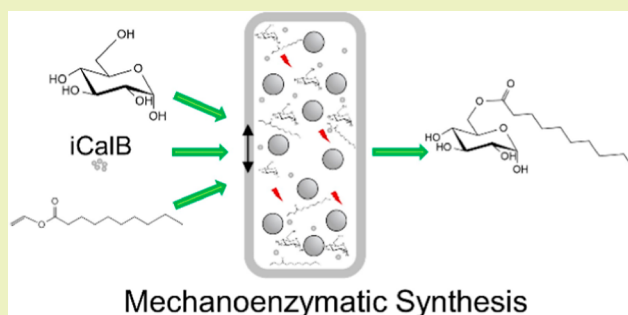
INTRODUCTION

Surfactants are part of our everyday life as they occur in cosmetics, pharmaceuticals, foods, petrochemicals, mining, metallurgy, agrochemicals, fertilizers, and so forth.[1,2] Yet most are produced out of crude oil, which means that they rely on a limited resource. In 2017, surfactants had an annual global revenue of USD 43.6 billion.[3] The prices of surfactants are suspected to increase because of the high variability in the oil price in recent years as well as due to higher demand.[4] An environmentally friendlier alternative to fill the gap of this ending resource could be the use of biosurfactants. In Europe alone, biobased surfactants had an annual growth rate of 3% between 2008 and 2013. Thus, sustainable synthesis routes are needed for the production of glycolipids.[5] Moreover, the sales of biosurfactants worldwide are predicted to reach revenue of up to USD 5.52 billion in 2022. As a flagrant example, rhamnolipids alone are foretold to reach an 8% sales growth until 2023.[6] Biosurfactants and more particularly glycolipids are sustainable, odorless, nontoxic, and tasteless compounds[5,7] characterized by mono-, di-, or oligosaccharides as their hydrophilic head moiety. The hydrophobic tail usually consists of one or more alkyl moieties with varying chain lengths. This versatility leads to a wide variety of glycolipids.[8] Moreover, the ester bond between the two building blocks of glycolipids

makes them inherently degradable in nature, hence limiting the risk of harmful accumulations in the environment. Glycolipids, like rhamnolipids, sophorolipids, or mannosylerythritol lipids, have been reported to be bioactive: for example, sophorolipids show anti-inflammatory effects in vitro as well as in vivo;[9−11] rhamnolipids improve wound healing in rats[9,12,13] and inhibit biofilm formation.[9,14,15] They can be also effective against certain viruses and fungi and are potential anti-tumor agents.[3,16] Moreover, glycolipids are highly surface-active' they efficiently reduce surface tension, exhibit a low critical micelle concentration, and act as foam stabilizers.[9,17] Consequently, these components gained rapid interest for industrialization purposes. Thus, at the industrial scale, the production of glycolipids such as sugar esters is mostly done by chemical synthesis that is cost-effective with reasonably high yields. However, the chemical procedure requires the use of harsh conditions like high temperature, acidic catalysis (e.g., by
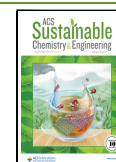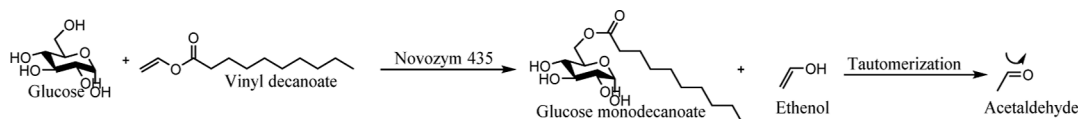
Scheme 1. Reaction Scheme to Produce Glucose Monodecanoate

perfluorinated alkylsulfonic acid), and complicated processing requiring multiple steps of protection, deprotection, and activation (e.g., with allyl, benzyl, and *p*-methoxybenzyl groups), leading to wasteful side products.[18−20] Nonetheless, these compounds are of great interest in the food-, cosmetic-, and pharmaceutical industry. For example, sorbitol-6-*O*-laurate, a model compound studied by Delavault et al.,[21] is structurally related to an emulsifier registered as E493, which is commonly employed for pharmaceutical and food applications and approved by the European Food Safety Authority (EFSA).[22] Enzymatic synthesis of glycolipids provides an alternative to chemical synthesis as milder conditions are applicable, for example, relatively low temperature, as well as selective synthesis, which reduces side product formation.[23] However, enzymatic glycolipid synthesis faces some challenges as the substrates show a wide range of polarity and simultaneous solubilization of polar sugars and non-polar fatty acids is difficult.[24,25]

Mechanochemistry is one of IUPACs 10 world-changing technologies and allows accelerated reaction velocities in solvent-free conditions.[26] First reported mechanoenzymatic reactions were enzymatic starch liquefaction and saccharification using amylases in a twin screw extruder.[27,28] One of the most common biocatalysts used in mechanoenzymology is the extensively studied Novozym 435, also known as the immobilized *Candida antarctica* lipase B (CALB).[29] It was used in the first mechanoenzymatic production of fine chemicals for the kinetic resolution of secondary alcohols by ball milling.[30] Kinetic resolution by mechanoenzymology was extensively studied as well, for many active pharmaceutical ingredients due to high enantioselectivity.[31] CALB was also applied in further mechanoenzymatic reactions, for example, acylation of primary amines, hydrolysis of amino esters, or ring-opening polymerization of *ω*-pentadecalactone.[32] Moreover, the use of organic solvents, volatile thus problematic in an eco-friendly context,[33] and extensive heat to reach the activation energy of certain chemical reactions can be reduced by using mechanochemistry.[34]

The objective of the present study is to evaluate the potential of mechanoenzymology to improve the performance of the biocatalyzed synthesis of glycolipids using a model reaction producing glucose monodecanoate (Scheme 1). Using a bead mill provided with glass beads, the transesterification of a sugar with a vinylated fatty acid was catalyzed by the commercial lipase formulation Novozym 435.

The synthesis was performed under conditions of reduced water content in order to reverse the hydrolysis activity of the lipase. Thus, organic solvents like 2-methyl-2-butanol (2M2B), ethyl acetate, and acetone were used alongside deep eutectic solvents (DES), as well as in solvent-free conditions. DES are non-toxic, non-volatile, and biodegradable and consist of hydrogen bond donors and hydrogen bond acceptors forming supramolecular structures with hydrogen bonding interactions, resulting in a liquid at room temperature.[35−37] In this regard, ubiquitous and naturally occurring plant metabolites may be used as hydrogen bond donors and acceptors, for example,

sugars, menthol, and choline chloride, among others. DES are reported to have stabilizing effects on enzymes and exhibit high dissolution power.[38−40] However, one of the major drawbacks of DES is their high viscosity compared to water and organic solvents.[25,41] The authors assumed that use of mechanoenzymology might overcome the drawback intrinsic to DES caused by high viscosity and the challenges of simultaneous substrate solubilization in different solvent systems. Indeed, successful use of mechanoenzymology was reported to be independent of solubility effects.[28,42] In the conducted experiments, hydrophobic DES made of menthol and decanoic acid were used.

■ MATERIALS AND METHODS

2M2B (purity ≥ 99%) and menthol (purity ≥ 99%), as well as acid washed glass beads (425−600 and 710−1180 *μ*m), were purchased from Sigma Aldrich Chemie GmbH (Taufkirchen, Germany). Glucose (purity ≥ 98%) and the solvents ethyl acetate (purity ≥ 99.5%) and acetone (purity ≥ 99.5%) were acquired from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). Lipase formulation, Novozym 435, was purchased from STREM Chemicals Europe (Bischheim, France). Glucose monodecanoate (purity > 99%) was acquired from Sohena (Tübingen, Germany). VD (purity > 99%) and decanoic acid (purity ≥ 98%) were acquired from Tokyo Chemical Industry Co., Ltd. (TCI Europe, Belgium). All chemicals were used without further purification.

Preparation of DES. The hydrophobic DES were prepared using an equimolar ratio of menthol and decanoic acid according to Hollenbach et al.[43]

Solvent Screening: Glycolipid Synthesis in the Bead Mill. For synthesis, 0.5 M glucose (50 mg), 0.5 M VD (65 *μ*L), and 20 mg Novozym 435 (33 mg/mL) were mixed in 1.5 mL Superspin microtubes (20170-030; VWR International GmbH, Darmstadt, Germany) with 0.5 g of glass beads (710−1180 *μ*m) and 600 *μ*L of solvent. The tubes containing the reaction mixture were incubated in a MM300 bead mill (Retsch GmbH, Haan, Germany) at 25 Hz (Figure 1). Experiments were performed in triplicates, and three tubes were collected for each time point (5, 10, 15, 30, 45, 60, and 90 min).

Yield, space-time yield, and biocatalyst productivity were calculated as follows

$$\text{yield} = \frac{n(\text{product})}{n(\text{glucose})} \times 100\%$$

(1)

Figure 1. Bead mill apparatus for processing the samples.

$$space - time\ yield = \frac{c(product)}{t} \tag{2}$$

$$biocatalyst\ productivity = \frac{n(product)}{t \times m(biocatalyst)} \tag{3}$$

where n is the number of moles in μmol, c the molar concentration in mM, t the operating time in h, and m the mass in g.

Influence of Milling Frequency, the Size of Glass Beads, and Pre-grinding of the Enzyme Formulation in 2M2B: Single Factor Experiments. Different frequencies (5, 10, 17.5, and 25 Hz) were tested in 2M2B at 45 min reaction time without varying any other reaction parameter to evaluate the optimal milling frequency.

To examine the influence of the bead size on the reaction, two different glass bead sizes (425−600 and 720−1180 μm) were tested at a reaction time of 45 min, while all other reaction conditions were kept constant (20 mg Novozym 435, 0.5 g of glass beads, 600 μL of solvent, 25 Hz).

To address the influence of ground enzyme on the reaction, Novozym 435 was pre-ground manually for approximately 10 min with the help of a ceramic mortar until a visibly fine powder was observed (Figure S1). The pre-ground enzyme had a Sauter diameter of 58.6 ± 0.8 μm (Table 1, Figure S2). The pre-ground enzyme was added to the reaction mixtures instead of whole Novozym 435 beads without changing any other parameter.

Table 1. Particle Size of Whole and Ground Enzyme

biocatalyst	whole Novozym 435	ground Novozym 435
Sauter diameter $d_{3,2}/\mu$m	598.7 ± 22.2	58.6 ± 0.8
$d_{10,3}/\mu$m	467.3 ± 18.2	24.7 ± 0.5
median $d_{50,3}/\mu$m	617.3 ± 22.5	202.2 ± 5.2
$d_{90,3}/\mu$m	794.1 ± 22.6	589.6 ± 17.7
span/μm	0.53 ± 0.02	2.79 ± 0.03

Energy consumption of the different methods was calculated as follows

$$energy\ consumption = \sum P \times t \tag{4}$$

where P is the power of the applied machines in kW and t is the operation time in h.

Influence of the Substrate Ratio. Different substrate ratios of glucose:VD were investigated such as the following: 0.5:0.5 M (1:1), 0.5:0.25 M (2:1), and 0.25:0.5 M (1:2) in 2M2B and in the hydrophobic menthol:decanoic acid DES. All other reaction conditions were kept constant [20 mg Novozym 435, 0.5 g of glass beads (710−1180 μm), 600 μL of solvent, 25 Hz, 90 min].

Influence of Enzyme Concentration. To examine the optimal enzyme concentration, different concentrations of Novozym 435, that is, 20, 33, 50, 100, and 200 mg/mL, were employed in 2M2B and the hydrophobic menthol/decanoic acid DES without varying any other reaction parameter [0.5 M glucose, 0.5 M VD, 0.5 g of glass beads (710−1180 μm), 600 μL of solvent, 25 Hz, 90 min].

Solvent-Free (Neat) Synthesis of Glycolipids Using a Bead Mill Apparatus. To investigate the mechanoenzymatic synthesis in a solvent-free system, 50 mg of glucose and 20 mg Novozym 435 were mixed in superspin tubes with 0.5 g of glass beads (710−1180 μm) alongside different volumes of VD: 33, 65, 130, 260, and 520 μL as both reactants and adjuvants. Samples were incubated for 90 min at 25 Hz in the bead mill.

Glycolipid Synthesis Using Conventional Heating. As a reference, a standard heating method was used. Therefore, the reaction was carried in 2M2B at 50 °C in a rotator with a vortex mixer (program U2) from neoLab (Heidelberg, Germany), which enables thorough mixing of liquid samples in tubes by vertical rotation and vibration, at 90 rpm for 90 min within an oven, with or without glass beads to allow for further comparison between heating methods after data treatment. The same reaction vessels were used as in the mechanoenzymatic synthesis, and the solvent volume of 600 μL was

also retained as well as the other reaction conditions [0.5 M glucose, 0.5 M VD, 20 mg Novozym 435, 0.5 g of glass beads (710−1180 μm), 600 μL of solvent, 25 Hz, 90 min].

Quantification of Glycolipids. Samples were centrifuged after the synthesis. Tubes containing organic solvents were treated as follows: 0.2 mL of the supernatant was transferred into a 1.5 mL tube and dry-evaporated using a SpeedVac centrifugal evaporator (H. Saur Laborbedarf, Reutlingen, Germany) and resuspended in 0.2 mL of ethyl acetate, then subsequently analyzed by high-performance liquid chromatography (HPLC).

For tubes containing DES, samples were diluted with ethyl acetate (1:1, v/v) and analyzed by HPLC. Glycolipids were quantified by HPLC-evaporative light scattering detector (ELSD) as described by Hollenbach et al.[25] Briefly, reversed-phase chromatography was performed using a Kinetex EVO C18 (2.6 μm, 250 × 4.6 mm) from Phenomenex (Aschaffenburg, Germany) with an accompanying guard column (4 × 3.0 mm ID) of the same phase and an Agilent (Germany) 1260 series liquid chromatograph equipped with a quaternary pump, an autosampler, and a column oven. An ELSD from BÜCHI Labortechnik (Essen, Germany) was operated at 38 °C, a gas flow rate (air) of 1.5 mL/min, and a gain of 1. Mobile phase was a gradient of acetonitrile (A) and water (B) with a total flow rate of 1 mL/min. The gradient started from 40% A-60% B; then from 0 to 10 min, there was a linear gradient up to 35% A-65% B; followed by another linear gradient from 10 to 15 min up to 25% A-75% B. This gradient was held for 5 min, followed by a reconditioning step of the column with 40% A-60% B for 5 min. Limit of detection was 0.0014 mM.[25] A commercially available standard of glucose monodecanoate was used for calibration.

Solvent-free samples were diluted with 600 μL of ethyl acetate and thereafter analyzed by HPLC-ELSD.

Structural Elucidation of Glucose Monodecanoate. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AVANCE II+ 600 MHz spectrometer (Bruker BioSpin, Rheinstetten, Germany) equipped with a BBI probe head at 300 K. Prior to analysis, 10 mg glucose monodecanoate was dissolved in 0.6 mL CD_2Cl_2/d_6-acetone (4:1, by vol). For structural elucidation, 1D ^{1}H NMR spectroscopy and 2D ^{1}H−^{1}H correlation spectroscopy (COSY), ^{1}H−^{13}C heteronuclear single−quantum correlation (HSQC) spectroscopy, and ^{1}H−^{13}C-heteronuclear multiple-bond correlation (HMBC) spectroscopy were performed. Spectra were processed and analyzed using Topspin 4.1.4 (Bruker BioSpin) and MestReNova 14.2.0 (Mestrelab Research S.L., Santiago de Compostela, Spain). Chemical shifts were reported relative to the ^{1}H and ^{13}C resonance of tetramethylsilane.

One- and two-dimensional NMR experiments were performed to get a detailed structural description of glucose monodecanoate (Figures S3−S6). Two ring systems were identified in ^{1}H−^{1}H COSY, ^{1}H−^{13}C HSQC, and ^{1}H−^{13}C HMBC spectra that can be assigned to the α- and β-anomers of glucose (Table S1). The decanoate chain could be traced in ^{1}H−^{1}H COSY and ^{1}H−^{13}C HMBC spectra. Based on the cross-peaks between glucose protons and the lipid carbonyl in the ^{1}H−^{13}C HMBC spectrum, the position of the acylation could be confirmed. Both glucose anomers were acylated on C6.

The mass spectrometry (MS) for mass identifications was performed with electrospray ionization on a quadrupole Q Exactive Plus (Thermo Fisher Scientific GmbH, Kandel, Germany) and recorded in a positive mode. The raw spectrometric data were treated using MestReNova Suite 2020 (version 14.2.0) (Mestrelab Research S.L., Santiago de Compostela, Spain). m/z 282.279 $[M_{GMD} + H]^+$-3H_2O; m/z 299.185 $[M_{GMD} + H]^+$-2H_2O; m/z 317.196 $[M_{GMD} + H]^+$-H_2O; m/z 335.206 $[M_{GMD} + H]^+$; m/z 352.233 $[M_{GMD} + NH_4]^+$; m/z 357.188 $[M_{GMD} + Na]^+$ (Table S2 and Figure S7).

Particle Size Distribution Measurements. The particle size distribution of the whole and ground enzyme formulation was characterized using a Horiba LA-940 laser diffraction particle analyzer from Retsch Technology GmbH (Haan, Germany).

The pictures were acquired and scaled using a microscope Nikon Eclipse E200 coupled to an NIS Elements D version 4.50 software, both purchased from Nikon Germany (Düsseldorf). For illumination,

the spotlight of a KL 1500 LCD goose neck lamp from Schott AG (Mainz, Germany) was placed on the left and right sides of customized glass slides.

Data Treatment and Statistical Analysis. OriginPro software 9.7 (version 2020) (OriginLab Corporation, Northampton, MA, USA) was used for raw data treatment and statistical analysis. Results are presented as mean ± standard deviation ($n = 3$). Statistical analysis was performed by one-way ANOVA and Tukey test, and the results were considered significant if the p-value was <0.05 if not stated otherwise.

RESULTS

The present work investigates for the first time the synthesis of a glycolipid, namely, a sugar ester, assisted by a mechanoenzymatic method in both dissolved and neat reaction systems. Glucose monodecanoate was produced using a bead mill, and the efficiency was compared to conventional convective thermal heating. For the bead mill-assisted synthesis, different solvent systems were evaluated to mediate the quasi-simultaneous transesterification/esterification reactions.[44] Thus, by following the product formation over time, different parameters such as substrate ratio, milling frequency, and enzyme concentration were optimized. Finally, a solvent-free approach was presented and compared to the dissolved reaction systems made of either organic solvent (2M2B) or hydrophobic DES that can both be seen as unconventional media for a biocatalyzed reaction.

Influence of Different Solvents. Figure 2 shows the reaction time course of the mechanoenzymatic glycolipid
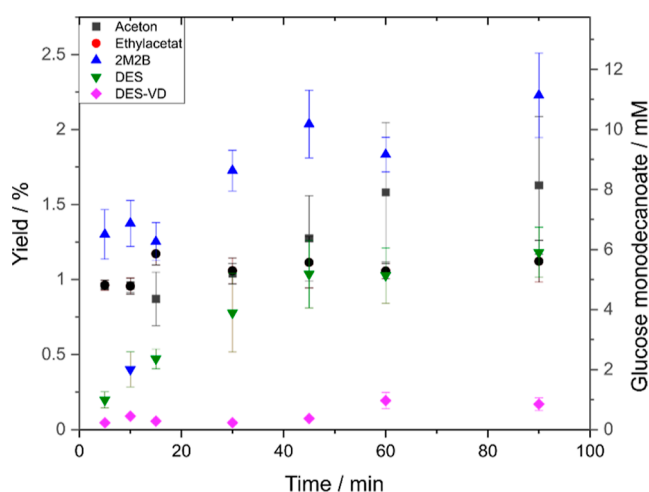

Figure 2. Influence of different solvents on the mechanoenzymatic synthesis of glucose monodecanoate. Highest product titers were achieved in 2M2B. Reaction conditions: substrate ratio glucose: VD is 1:1 (0.5/0.5 M); 33 mg/mL Novozym 435; 0.5 g of glass beads (710−1180 μm); 600 μL of solvent; bead milling frequency: 25 Hz. 2M2B: 2-methyl-2-butanol; DES: hydrophobic (−)-menthol: decanoic acid DES; DES-VD: hydrophobic (−)-menthol: decanoic acid DES without addition of VD. $n = 3$, p-value < 0.05.

synthesis in different solvents. Highest yields were obtained in 2M2B (2.2 ± 0.3% corresponding to 11.1 ± 1.4 mM), followed by acetone (1.6 ± 0.4% corresponding to 8.1 ± 2.3 mM). Synthesis in ethyl acetate (1.1 ± 0.2% corresponding to 5.6 ± 0.7 mM) and the hydrophobic (−)-menthol:decanoic acid DES (1.2 ± 0.2% corresponding to 5.9 ± 0.8 mM) resulted in similar yields. Notably, production of glucose monodecanoate in DES without addition of activated fatty acid

resulted in considerably lower yields (0.2 ± 0.05% corresponding to 0.8 ± 0.2 mM) in comparison to those achieved with the addition of VD in organic solvents and DES.

Comparison of Mechanoenzymatic Reactions to Conventional Heating. Samples were incubated in an oven provided with an overhead shaker with vortex mixer to compare the reaction yields of the mechanoenzymatic reaction (Table 2).

Table 2. Comparison of Glucose Monodecanoate by Mechanochemistry Using a Bead Mill vs Conventional Convective Heating after 90 min of Processing

solvent	condition[a]	glucose monodecanoate/mM[b]	yield/%
2M2B	oven w/o glass beads	8.7 ± 0.6	1.7 ± 0.1
	oven w/glass beads	11.5 ± 0.4	2.3 ± 0.1
	bead mill (25 Hz)	11.1 ± 1.4	2.2 ± 0.3
DES	oven w/o glass beads	4.3 ± 0.2	0.9 ± 0.05
	oven w/glass beads	4.4 ± 0.5	0.9 ± 0.1
	bead mill (25 Hz)	5.9 ± 0.8	1.2 ± 0.2

[a]Oven was used as the standard heating method used as reference at 50 °C provided w/(with) or w/o (without) glass beads in the reaction vessel. [b]Data are presented as mean values ± standard deviations ($n = 3$).

Glycolipid titers reached after 90 min without the addition of glass beads were lower in both solvents when using a conventional heating method compared to the bead mill-assisted synthesis. In addition, experiments were performed in which glass beads were added to the reaction vessel but processed by conventional heating instead of the bead mill. These experiments were performed to evaluate whether the lower mechanical forces reached in a shaker provided with glass beads could also reach an intensification of the process similar to the effect observed in the proper bead mill apparatus. Using 2M2B, the initial glycolipid concentration was similar between the mechanoenzymatic approach and the conventional heating method when glass beads were added to the reaction tubes. For the synthesis in DES, however, the addition of glass beads in combination with conventional heating did not achieve the glycolipid titers reached with the bead mill. Furthermore, the synthesis in the bead mill resulted not only in higher product concentration but also in lower energy consumption. The operation of the ball mill consumed 0.27 kW h within the reaction time of 90 min, whereas the energy consumption of the shaker and heating oven was 0.99 kW h.

Influence of Frequency, Pre-grinding of Enzyme Formulation, and Glass Bead Size. Herein, 2M2B was used as a solvent and the reaction time was set to 45 min. The influence of the milling frequency as well as the effect of pre-grinding on the enzyme activity was evaluated. For whole enzyme formulation, the product concentration increased with an increase in shaking frequency up to the highest one allowed by the milling apparatus (Figure 3). Using pre-ground enzyme formulation, a frequency of 17.5 Hz resulted in similar yields as 25 Hz. Consequently, the milling frequency could be reduced when a pre-ground enzyme formulation was used accordingly. However, the maximal product titers reached could not be increased by the pre-grinding.

Two different sizes of glass beads were investigated in this study, that is, 425−600 and 720−1180 μm of diameter. The bigger beads resulted in a slightly higher product titer (10.2 ±
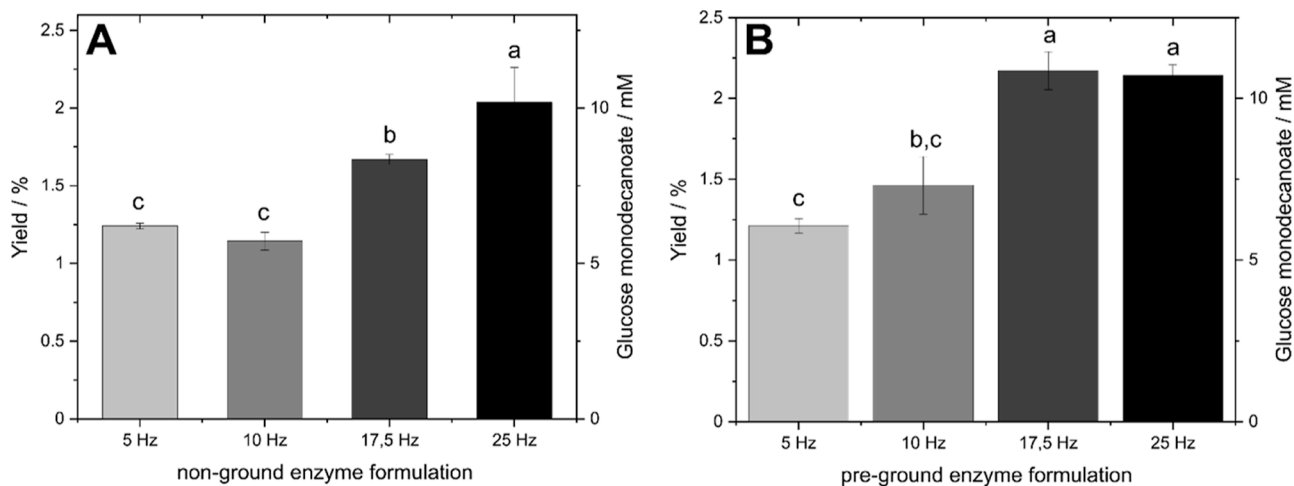
Figure 3. Influence of the milling frequency and a pre-grinding step on mechanoenzymatic glycolipid production yields. Reaction conditions: substrate ratio glucose/VD is 1:1 (0.5/0.5 M) at a reaction time of 45 min; 33 mg/mL Novozym 435; 0.5 g glass beads (710−1180 μm); 600 μL of solvent. (a−c) Statistically significant differences ($p < 0.05$).
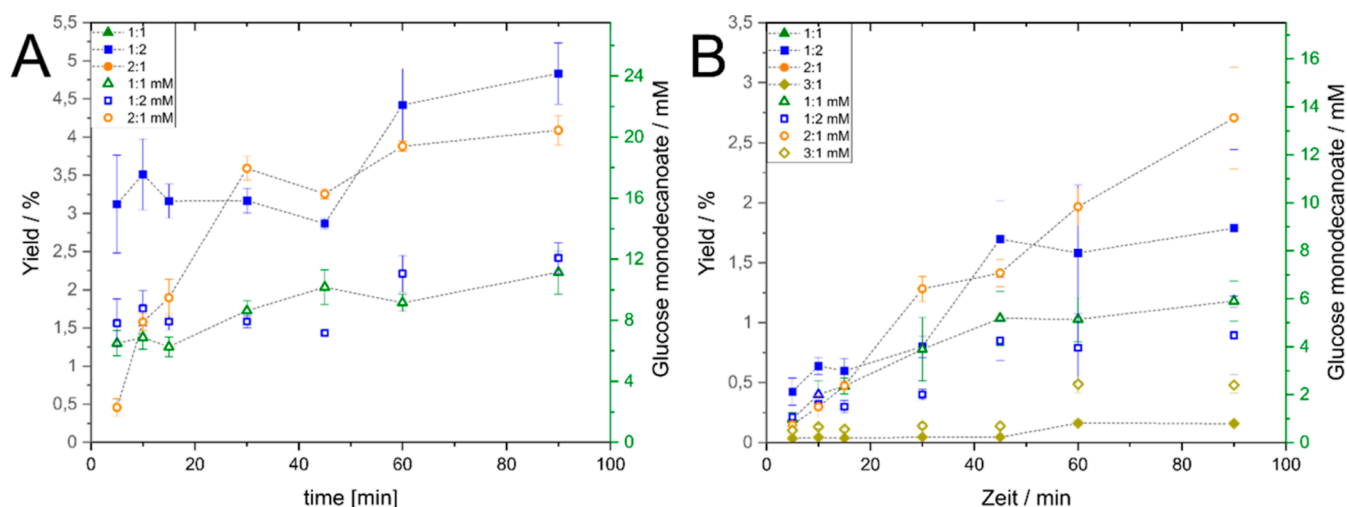

Figure 4. Influence of substrate ratio on product titers and yields. (A) In 2M2B; (B) in (−)-menthol:decanoic acid DES. Tested ratios: 1:1 (0.5 M glucose: 0.5 M VD), 1:2 (0.25 M glucose: 0.5 M VD), and 2:1 (0.5 M glucose: 0.25 M VD). For equimolar ratio and 2:1, the curves of yield and concentration are overlapping. Reaction conditions: 33 mg/mL of Novozym 435; 0.5 glass beads (710−1180 μm); 600 μL of solvent; 25 Hz milling frequency. Data are presented as mean values $\pm$ standard deviations ($n = 3$).
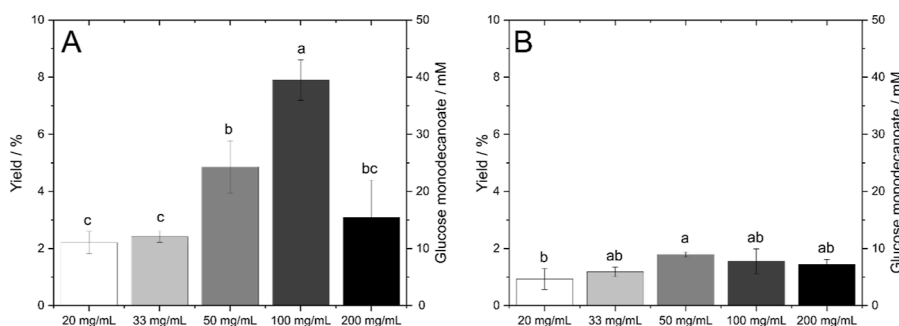

Figure 5. Influence of enzyme loading on mechanoenzymatic production of glucose monodecanoate. (A) In 2M2B; (B) in DES. Reaction conditions: substrate ratio glucose: VD is 1:1 (0.5/0.5 M); 0.5 g glass beads (710−1180 μm); 600 μL solvent; 25 Hz milling frequency; 90 min. (a−c) Statistically significant differences ($p < 0.05$).

1.1 mM) compared to the smaller beads (9.2 $\pm$ 0.8 mM). However, this difference was not statistically significant.

Influence of Substrate Ratio. The substrate ratio was accessed as a reaction parameter for optimization in 2M2B as

well as in DES. For both solvent systems, a substrate ratio of 2:1 (glucose:VD) resulted in significantly higher glycolipid concentrations than the other substrate ratios investigated (Figure 4). However, in terms of yields, this observation was
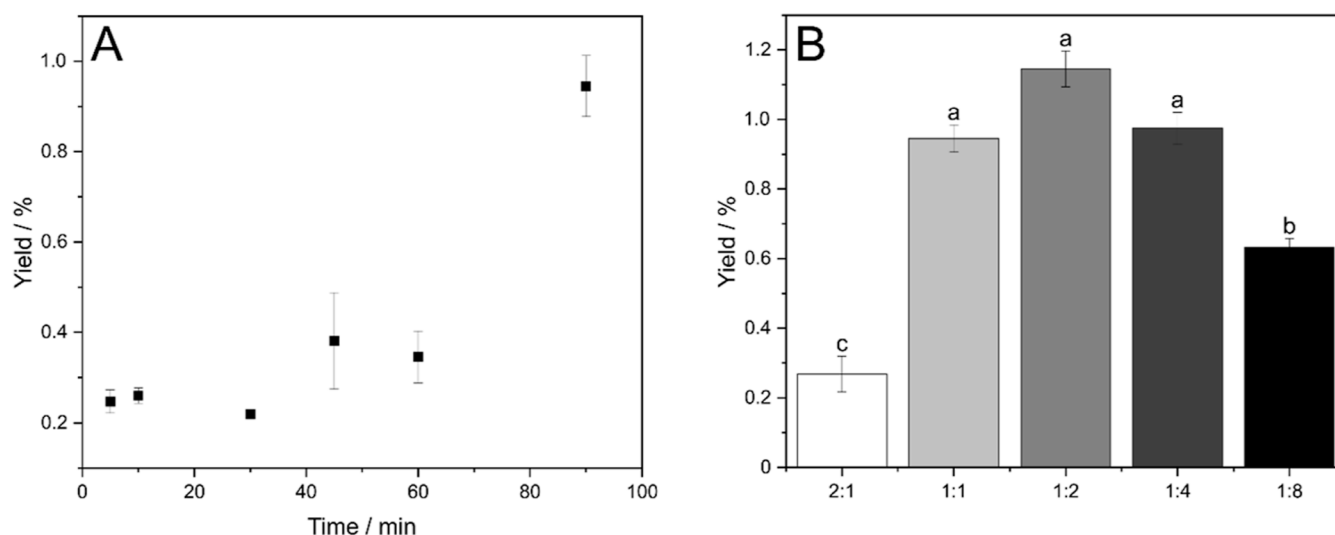
Figure 6. Solvent-free glycolipid synthesis. (A) Reaction time course of glucose monodecanoate production in a solvent-free mechanoenzymatic synthesis (substrate ratio glucose:VD is 1:1). (B) Influence of VD ratio on glucose monodecanoate yields in solvent-free mechanoenzymatic synthesis (substrate ratio glucose:VD is 2:1; 1:1; 1:2; 1:4; 1:8). Reaction conditions: 20 mg Novozym 435; 50 mg glucose; 0.5 g glass beads (710−1180 μm); 90 min, 25 Hz milling frequency. (a−c) Statistically significant differences ($p < 0.05$).

non-significant in DES, and in 2M2B, the yield was the highest at a substrate ratio of 1:2. In DES, the product concentration was slightly higher for an equimolar ratio than for a 1:2 ratio. However, there was no statistically significant differences between an equimolar ratio and a substrate ratio of 1:2. In 2M2B, the yields achieved for a substrate ratio of 1:2 were significantly higher compared to the equimolar ratio.

Influence of Enzyme Loading. In order to increase the productivity of the mechanoenzymatic process, different enzyme concentrations were tested (Figure 5). Glucose monodecanoate concentration increased with increasing enzyme concentration up to 100 mg/mL in 2M2B and 50 mg/mL in DES. At higher enzyme concentrations, the product titers decreased. The influence of the enzyme concentration was more pronounced in 2M2B than in DES. For the mechanoenzymatic synthesis in 2M2B, the glycolipid concentration was quadrupled (from 11.9 ± 1.9 mM at 20 mg/mL to 39.5 ± 3.5 mM at 100 mg/mL), while the product concentration in DES was only doubled with optimized enzyme concentration (from 4.6 ± 1.8 mM at 20 mg/mL to 9.0 ± 0.4 mM at 50 mg/mL).

Solvent-Free Synthesis. Neat reaction systems, also known as solvent-free systems, have drawn considerable attention over the past decades as they allow almost complete riddance of solvents and thus simplify waste management strategies.[45,46] Their use in mechanoenzymology is highly relevant as liquid substrates; for example, fatty esters in our case, can play the role of an adjuvant to enhance mass transfer. With those considerations, neat systems are therefore a tool of high interest for green chemistry and more particularly biocatalysis. It therefore appeared logical to investigate a solvent-free method in the frame of the mechanoenzymatic glycolipid synthesis.

Figure 6A shows the reaction time course. The data indicate that after the reaction time of 90 min, a steady state is not yet reached. However, longer reaction times could not be addressed due to limitations in the maximal operation time of the milling apparatus.

Different volumes of VD were evaluated in this study. The yield of glucose monodecanoate was the best at a slight excess of C10 vinylated fatty ester or equimolar ratio of the substrates (Figure 6B). Upon 520 μL of VD, an inhibition of the reaction was observed. The maximum achieved yield in the solvent-free system was 1.1 ± 0.07%, while in 2M2B, yields up to 11.4 ± 2.4% and in DES up to 2.0 ± 0.2% were reached.

■ DISCUSSION

In this study, a mechanoenzymatic approach for glycolipid synthesis was evaluated. Mechanochemistry has been widely applied in various fields of chemistry;[47−50] however, modern mechanochemistry has emerged only recently in biocatalysis, as first reported in 2016.[30,48] We demonstrated the suitability of mechanoenzymatic glycolipid synthesis in two different unconventional dissolved reaction solvents such as organic solvents and hydrophobic DES. Most generally and until now, glycolipids have been produced in various organic solvents using conventional heating.[51−56] By means of mechanoenzymatic synthesis, yields were the highest in 2M2B, also known as tert-amyl alcohol, which is a solvent reported to be compatible with food and pharmaceutical applications.[57] With a log P of 1.1, 2M2B is less polar than the other investigated organic solvents, ethyl acetate, and acetone, which turns out to be valuable for biocatalysis as solvents of medium polarity are most suitable for glycolipid synthesis by allowing sugar dissolution without hindering enzyme activity.[43,52,53,58] Indeed, highly polar solvents can strip off water from the essential hydration shell of the enzyme, ending up in partial or complete denaturation of the latter.[59−61]

The mechanoenzymatic synthesis was compared to a conventional heating and mixing method. Higher product titers and yields were reached in the bead mill than in the conventional method. Additionally, the synthesis using conventional heating was performed with adjunction of glass beads in the reaction vessel and reached similar glycolipid concentrations as in the bead mill. This observation was made significant using 2M2B, while only minor effects were observed in DES. This indicates that in DES, the influence of shear

Table 3. Yield and Space-Time Yield of Enzymatic Glycolipid Production by Different Methods in Different Solvent Systems

method	solvent (n/n^a; enzyme quantity; biocatalyst)	reaction time/h	yield/%[c]	space-time yield/mM/h[c]	biocatalyst productivity/$\mu mol_{product}$/h/$mg_{biocatalyst}$	refs
BM	2M2B (1:1; 33 mg/mL, Novozym 435)	0.75	2.0 ± 0.2	13.5 ± 1.5	0.27 ± 0.03	this work
	2M2B (1:2; 100 mg/mL; Novozym 435)	1.5	11.4 ± 2.4	19.0 ± 3.9	0.13 ± 0.03	this work
	2M2B (1:1; 100 mg/mL Novozym 435)	1.5	7.9 ± 0.7	26.3 ± 2.3	0.17 ± 0.01	this work
	2M2B (2:1; 33 mg/mL; Novozym 435)	1.5	4.1 ± 0.2	13.6 ± 0.6	0.27 ± 0.01	this work
	DES (2:1; 33 mg/mL; Novozym 435)	1.0	2.0 ± 0.2	6.6 ± 0.5	0.13 ± 0.01	this work
	Solvent-free(1:2; 20 mg; Novozym 435)	1.5	1.1 ± 0.07	27.0 ± 1.9	0.09 ± 0.006	this work
conv	2M2B	1.5	1.9 ± 0.2	6.3 ± 0.8	0.12 ± 0.02	this work
	DES	1.5	0.9 ± 0.05	2.9 ± 0.15	0.06 ± 0.003	this work
conv.	DES (3:1; 20 mg/mL; Novozym 435)	24	11	6.8	0.34	43
conv.	2M2B (1:1; 10% SP 435)	72	55	6.4	0.06	52[b]
conv.	IL/t-butanol (1:2; 5% Candida antarctica lipase B)	72	60	4.2	0.08	62[b]

[a]Unless indicated, the molar ratio n/n corresponds to the glucose and VD. [b]In those studies, esters of sugars with slight structural variations were synthesized. [c]Averaged values.

mechanical forces was not as prevalent as in 2M2B when adding glass beads to the reaction mixtures to enhance the conversion. This conjecture is valid whether the synthesis was assisted by the heated overhead shaker or the bead mill. The difference in behavior of the reaction in 2M2B and DES is most likely due to the higher viscosity of the latter, which is known to affect mass transfer directly. The improved performances of the mechanoenzymatic reaction might be due to an increase in interface energy and decrease in activation energy, which is well-known for mechanochemistry.[50] These effects allow for high space-time yields and thus save a considerable amount of time. The comparison of our study with glycolipid syntheses described in the literature confirms higher space-time yields by mechanoenzymatic synthesis, which is 2−6 times higher than the reported productivity for sugar ester synthesis in the literature (Table 3). However, the comparison also shows that the yields in the study presented here are lower despite much greater space-time yields because no steady state was reached prior to attaining 90 min of reaction time. Hence, longer reaction times and temperature control will most likely increase the yields of the mechanoenzymatic reaction further. Unfortunately, this could not be investigated in this study due to a limited maximum running time of the ball mill used to prevent overheating of the unit.

Furthermore, the effect of enzyme formulation pre-grinding was accessed. Increased activity of ground immobilized *C. antarctica* lipase B was previously reported in conventional heating as well as in mechanoenzymology.[63,64] We observed that for the ground enzyme formulation, a lower milling frequency during the mechanoenzymatic reaction was necessary to reach the same product concentrations. The described effect is likely attributed to diffusional effects, because a higher surface of the biocatalyst carrier and a better distribution in the reaction mixture is achieved by grinding as the particles get smaller. The Sauter diameter of the biocatalyst particles was 10 times smaller for the ground enzyme formulation than for the whole one, thus adding evidence to our conjecture.

For conventional convective heating, a high amount of dissolved sugar proved to be beneficial for enzymatic glycolipid synthesis in different solvent systems.[24,43,65−67] Likewise, this study revealed a higher product concentration in the presence of glucose excess. The best substrate ratio was 2:1 (glucose:VD, n/n) for 2M2B as well as for DES.

To enhance product concentration, the biocatalyst loading was evaluated, resulting in an optimal enzyme concentration of 100 mg/mL in 2M2B and 50 mg/mL in DES. Higher enzyme loading resulted in a significant decrease in glycolipid concentration, which is also reported for other transesterification reactions.[43,68−70] On the one hand, this observation might be due to product hydrolysis by the excess enzyme and the water formed as a by-product.[43,44] On the other hand, a high enzyme excess could affect mass transfer, as a higher enzyme concentration correlates to a higher observed viscosity of the reaction mixture. In this study, samples with 200 g/L enzyme formulation load displayed a paste-like behavior after ball mill application and could no longer be associated to a proper liquid like the other samples. Optimal enzyme formulation concentrations of 50 mg/mL and higher are not uncommon with Novozym 435. For example, Delavault et al. revealed 50 mg/mL as ideal for the synthesis of sorbitol laurate[21] and enzyme concentrations of, for example, 150 mg/mL solvent or 190 mg per mmol substrate, have also been reported in mechanoenzymatic reactions.[68,71] Hereby, it is to consider that in the case of enzymes immobilized on beads, the non-catalytic material makes up a large part of the total mass (90 to >99 wt %).[72,73] For Novozym 435, a protein content of 30−55 mg/g is reported in the literature.[74] Therefore, 100 mg/mL Novozym 435 corresponds to only 3−5 mg/mL of protein. Moreover, Novozym 435 is a highly stable lipase formulation, and the reusability of this biocatalyst has been proven also in mechanoenzymatic reactions, reducing the enzyme costs in each consecutive cycle.[30,64,75] However, a valid recycling strategy has to be developed and evaluated to ensure economic applicability of such a process.

Solvent-free strategies are gaining interest as they enable more sustainable production by preventing the formation of wastes, increasing volumetric productivity, and accordingly minimizing downstream processing.[45,46,50,76−78] However, semisolid reaction mixtures thereby pose a huge challenge. In previous studies involving solvent-free syntheses of sugar esters, the practical difficulties in processing semisolid mixtures were addressed by high-pressure homogenization and addition of crude sugar esters or conditions under reduced pressures.[79,80] We did overcome the problem of immiscibility of the substrates, glucose and VD, owing to a mechanoenzymatic strategy.

In the solvent-free synthesis, we observed highest conversion employing a slight excess of fatty acid ester in contrast to the

dissolved reaction systems, using either 2M2B or DES, in which a slight excess of sugar performed best. This observation can be explained by the fact that the acyl acceptor, namely, glucose, is solid, while the vinyl fatty ester is liquid (at room temperature) and provides properties similar to those of an adjuvant. Thus, a higher amount of fatty acid leads to a higher proportion of liquid in the reaction mixture and hence to a better mixing. Still, an inhibitory effect was observed above a certain ratio of the fatty acid in solvent-free synthesis. It is known for different solvent systems that fatty acids inhibit glycolipid synthesis as well as different transesterification reactions.[55,81−83] Indeed, transesterification reactions follow a ping−pong bi bi mechanism and therefore the formation of non-productive complexes with either fatty acids or sugar is possible.[81,82,84]

CONCLUSIONS

The study provides first evidence for the applicability of mechanoenzymatic reactions in glycolipid production. We have shown that lipase-mediated glycolipid synthesis is possible in organic solvents as well as in DES using the mechanical shear forces provided by a bead mill apparatus. We achieved considerably higher space-time yields by mechanoenzymatic synthesis compared to a variety of conventional heating and mixing systems. Therefore, the mechanoenzymatic approach is most likely a promising strategy for glycolipid synthesis and biocatalysis as a whole, to save time and energy.

Moreover, we presented a solvent-free enzymatic synthesis for a glucose fatty acid ester for the first time, showing that the challenges of a semisolid system could be successfully overcome by a mechanoenzymatic approach.

ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssuschemeng.2c01727.

> Microscopical images of ground and whole Novozym 435; cumulative particle size distribution of ground and whole Novozym 435; NMR spectra of glucose monodecanoate; mass spectrum of glucose monodecanoate; chemical shifts obtained by NMR of glucose monodecanoate; and results of MS of glucose monodecanoate (PDF)

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R.H. and A.D. contributed equally. This study was conceptualized by R.H. and A.D. Investigations were performed by R.H., A.D., and L.G. NMR analysis was performed by H.S. and C.M.-G. The work was supervised by C.S. and K.O. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

DES, deep eutectic solvents; VD, vinyl decanoate; 2M2B, 2-methyl-2-butanol; ELSD, evaporative light scattering detector

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