Glycolipids for foaming applications
- from synthesis to structure-function relationship -

Zur Erlangung des akademischen Grades einer

DOKTORIN DER NATURWISSENSCHAFTEN

von der KIT-Fakultät für Chemieingenieurwesen und Verfahrenstechnik des Karlsruher Instituts für Technologie (KIT)
genehmigte

DISSERTATION

von

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Tag der mündlichen Prüfung: 30.03.2021

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It always seems impossible,
until it's done.
(Nelson Mandela)

Acknowledgements

I would like to express my sincere gratitude to all people who helped me all the way through my PhD study and contributed to the success of this work. I am very grateful for your support.

First and foremost, I would like to thank my supervisor Prof. Dr. Christoph Syldatk for the opportunity to work in his group, for his guidance, support and helpful comments, as well as for entrusting me with presentations at project meetings and international conferences.

I would like to thank everyone at TeBi. It was a pleasure to work with you in this relaxed and friendly atmosphere at TeBi. I am very grateful for your friendship, support, motivation and lots of cakes ☺ that helped to survive even the "bad" days. However, some deserve special mention:

- Olga (for being an amazing colleague and friend and especially for all the little lab stuff where you helped me during Corona times)
- Teresa (for a great time and lots of fun)
- Alba (for always listening, making everyone smile and editing some English issues)
- Andre (for all the discussions, good cooperation and the smooth coordination of the use of the equipment)
- the technician team, especially Pascal (for your help with orders, the lyophylle, HPLC 6 (and her "Brigitte"), the ELSD which drove us crazy especially at the beginning and for running to the storage room hundreds of times for solvent replenishment (Büchi and HPLC were sometimes incredibly thirsty))
- Susanne and Beate (for your help and advice with lots of formalities and business trip accounts)
- Sascha (for the guidance at the beginning of my PhD)
- Katrin (for input and proof-reading)
- Jens (for establishing the contact to MVM and input)

I want to thank my project partners outside of TeBi, from MVM, LVT and OC, for support with devices and scientific input, especially Dr. Ulrike S. van der Schaaf, Annika Völp and Prof. Dr. Norbert Willenbacher. Thanks to Prof. Dr. Norbert Willenbacher also for his kind acceptance for the co-referee.
I am grateful for the financial support by the European Regional Development Fund and the Ministry of Science, Research and the Arts of the State of Baden-Württemberg within the research center ZAFH InSeL.
I would like to express my gratitude to PD Dr. Karlis Briviba, Dr. Andrea Gille, Elisabeth Saftig and Konstanze Schelm, I owe my presentation confidence to you. Thanks that you made me enjoy all my talks no matter how big the audience was ☺
Jonas, thank you for your support, your patience and your understanding, as well as for making your computer corner an ideal workspace for HomeOffice and for ensuring that my laptop was always ready for any challenge.
Last, but not least I want to thank my family for their continuous support and encouragement. This thesis would not have been possible without you.
A very special thanks goes to Kyrill, thank you my four-legged friend, that you are always there for me and provide the perfect balance to my research.

Preamble

Parts of this thesis are based on peer-reviewed research articles. All articles have been drafted during this work and describe the major findings of the investigations of glycolipid synthesis in deep eutectic solvents and their foaming abilities. Chapters which have been previously published are indicated as such at the beginning of the chapter. The text of these chapters is partly identical to the publication. Layout, citation style and figures have been modified in order to match the formatting of this thesis.

Chapter 1 describes the theoretical background and is based on the publication:

Parameters influencing Lipase-Catalyzed Glycolipid Synthesis by (Trans-)Esterification Reaction
Rebecca Hollenbach, Katrin Ochsenreither, Christoph Syldatk
In: Advances in Biochemical Engineering/Biotechnology, Springer
Submitted

Chapter 2 presents the results of the optimization of glycolipid synthesis in hydrophilic deep eutectic solvents. This chapter is based on the publication:

Optimization of Glycolipid Synthesis in Hydrophilic Deep Eutectic Solvents
Rebecca Hollenbach, Benjamin Bindereif, Ulrike S. van der Schaaf, Katrin Ochsenreither, Christoph Syldatk
Frontiers in Biotechnology and Bioengineering, 2020, 8, 382. doi: 10.3389/fbioe.2020.00382

Chapter 3 outlines the applicability of a hydrophobic deep eutectic solvent for enzymatic glycolipid synthesis and the advantages of that particular hydrophobic deep eutectic solvent over hydrophilic deep eutectic solvents. This chapter is based on the publication:

Enzymatic Synthesis of Glucose Monodecanoate in a Hydrophobic Deep Eutectic Solvent
Rebecca Hollenbach, Katrin Ochsenreither, Christoph Syldatk
International Journal of Molecular Science, 2020, 21, 4342. doi: 10.3390/ijms21124342

Chapter 4 encompasses the investigation of structure-function relationship of glycolipids for foaming applications. This chapter is based on the publication:

Interfacial and Foaming Properties of Tailor-Made Glycolipids – Influence of the Hydrophilic Head Group and Functional Groups in the Hydrophobic Tail
Rebecca Hollenbach, Annika Völp, Ludwig Höfert, Jens Rudat, Katrin Ochsenreither, Norbert Willenbacher, Christoph Syldatk
Molecules, 2020, 25 (17), 3797, doi: 10.3390/molecules25173797

List of Publications

Peer reviewed original publications

Optimization of Glycolipid Synthesis in Hydrophilic Deep Eutectic Solvents
Rebecca Hollenbach, Benjamin Bindereif, Ulrike S. van der Schaaf, Katrin Ochsenreither, Christoph Syldatk
Frontiers in Biotechnology and Bioengineering, 2020, 8, 382. doi: 10.3389/fbioe.2020.00382

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Molecules, 2020, 25 (17), 3797, doi: 10.3390/molecules25173797

Evaluation of downstream processing, extraction, and quantifica-tion strategies for single cell oil produced by the oleaginous yeasts Saitozyma podzolica DSM 27192 and Apiotrichum porosum DSM 27194
Olga Gorte*, Rebecca Hollenbach*, Ioannis Papachristou, Christian Steinweg, Aude Silve, Wolfgang Frey, Christoph Syldatk and Katrin Ochsenreither
Frontiers in Bioengineering and Biotechnology. 2020, 8, 355. doi: 10.3389/fbioe.2020.00355
*Co-first authorship

Book chapter

Parameters influencing Lipase-Catalyzed Glycolipid Synthesis by (Trans-)Esterification Reaction
Rebecca Hollenbach, Katrin Ochsenreither, Christoph Syldatk
In: Advances in Biochemical Engineering/Biotechnology, Springer
Submitted

Poster presentations

Enzymatic Synthesis and Purification of Glycolipids
Rebecca Hollenbach, Christoph Syldatk
1. InSeL Symposium (2018), Reutlingen

Optimization of the extraction of glucose monodecanoate synthesized in a deep eutectic solvent by response surface modelling.
Rebecca Hollenbach, Christoph Syldatk
10th Workshop on Fats and Oils as Renewable Feedstock for Chemical Industry (2019), Karlsruhe.

Einsatz von Biotensiden zur Herstellung von Polymerschäumen.
Rebecca Hollenbach, Ludwig Höfert, Annika Völp, Christoph Syldatk
InSeL Evaluierung (2019), Pforzheim

Optimization of glycolipid synthesis in deep eutectic solvents and downstream processing.
Rebecca Hollenbach, Christoph Syldatk
Biosurfactants (2019), Hohenheim

Optimization of glycolipid synthesis in deep eutectic solvents (DES)
Rebecca Hollenbach, Katrin Ochsenreither, Christoph Syldatk
10. ProcessNet-Jahrestagung und 34. DECHEMA-Jahrestagung der Biotechnologen (2020), Web-Konferenz

Conference talks

Enzymatic Synthesis and Purification of Glycolipids
Rebecca Hollenbach, Christoph Syldatk
1. InSeL Symposium (2018), Reutlingen

Enzymatic synthesis and purification of glycolipids.
Rebecca Hollenbach, Christoph Syldatk.
2. InSeL Symposium (2019), Karlsruhe.

Strategies for the enzymatic synthesis of glycolipid surfactants – opportunities and challenges.
Rebecca Hollenbach, Christoph Syldatk
Biosurfactants (2019), Hohenheim

Structure-function relationship of glycolipids for the application of wet foams
Rebecca Hollenbach, Annika Völp, Katrin Ochsenreither, Norbert Willenbacher, Christoph Syldatk
6th Cellular Materials CellMAT 2020, Web-Konferenz

Interfacial and foaming properties of tailor-made glycolipids
Rebecca Hollenbach, Annika Völp, Katrin Ochsenreither, Jens Rudat, Norbert Willenbacher, Christoph Syldatk
International Conference on Biobased Surfactants Ghent (ICBS), Web-Konferenz

Abstract

Growing environmental awareness is leading to a quest for sustainable processes and sustainable products. Glycolipids are considered an alternative to petrochemically based surfactants because they are non-toxic, biodegradable, and less harmful to the environment while having comparable surface-active properties. They can be produced by microbial fermentation, as well as chemically or enzymatically in organic solvents or in deep eutectic solvents (DES) from renewable resources. Enzymatic synthesis enables the production of tailor-made glycolipids as a nearly unlimited number of glycolipids can be produced by combining different head and tail groups. Environmentally friendly and biodegradable reaction media are an important part of a sustainable glycolipid production in the transition to green chemistry. DES are an eco-friendly alternative to organic solvents, as DES are non-flammable, non-volatile, biodegradable, and almost non-toxic. Unlike organic solvents, sugars are readily soluble in hydrophilic DES. However, DES are highly viscous systems and restricted mass transfer is likely to be a major limiting factor for their application. Limiting factors for glycolipid synthesis in DES are generally not well understood.

Therefore, this thesis provides a quantitative analysis of glycolipid synthesis in hydrophilic DES whereby limiting factors are identified and an optimization strategy is derived (Chapter 2). Furthermore, hydrophobic DES are introduced as reaction media for enzymatic glycolipid production for the first time (Chapter 3). In a further step, the structure-function relationship of glycolipids for foaming applications was investigated in order to provide the basis for tailor-made production (Chapter 4).

In a first step, an HPLC method with ELS detection for quantitative analysis of reaction rates and yields was developed. This method allows for direct product quantification, so that indirect analysis via the consumption of fatty acid becomes unnecessary. Direct quantification offers the advantage that mono- and diesters can be distinguished, whereas this is not possible using the indirect method. The developed HPLC ELSD-method proved to be very sensitive, since the quantification limit was at a glycolipid concentration of 1.4 μM.

DES exhibit high viscosities compared to water and organic solvents. The investigated choline chloride:urea DES, for example, has a viscosity of 0.28 Pa·s at 20 °C and the choline chloride:glucose DES 1.41 Pa·s, while the viscosity of water is only 0.001 Pa·s. Thus, the influence of external mass transfer, fatty acid concentration, and distribution on initial reaction velocity in two hydrophilic DES was investigated. At agitation speeds of and higher than 60 rpm, the viscosity of both DES did not limit external mass transfer. Fatty acid concentration of 0.5 M resulted in highest initial reaction velocity while higher concentrations had negative effects. Fatty acid accessibility was identified as a limiting factor for glycolipid synthesis in hydrophilic DES. Mean droplet sizes of fatty acid-DES emulsions can be significantly decreased by ultrasonic treatment resulting in significantly increased initial reaction velocity and yield (from 0.15 ± 0.03 mmol glucose monodecanoate/g DES to 0.57 ± 0.03 mmol/g) in the choline chloride: urea DES. Physical pretreatment of fatty acid-DES emulsions proved to be suitable to improve the availability of fatty acids.

In order to eliminate the problem of limited fatty acid accessibility, the applicability of a hydrophobic DES was investigated. So far, only hydrophilic DESs were considered for

enzymatic glycolipid synthesis. In this thesis, a hydrophobic DES consisting of (-)-menthol and decanoic acid is presented for the first time as an alternative to hydrophilic DES. The yields in the newly introduced hydrophobic DES are significantly higher than in hydrophilic DES. Furthermore, both esterification and transesterification are possible in the (-)-menthol: decanoic acid-DES, thus the additional reaction step for activation of the fatty acid is no longer obligatory. Different reaction parameters were investigated to further optimize the synthesis. 20 mg/mL of lipase (Novozym 435) and 0.5 M glucose resulted in the highest initial reaction velocity for the esterification reaction, while the highest initial reaction velocity was achieved with 1.5 M glucose in the transesterification reaction. The enzyme was proven to be reusable for at least five cycles without significant loss of activity.

In a further step, investigations on the structure-function relationship of glycolipids were performed, as these analyses represent an essential step on the path to tailor-made glycolipid production. Interfacial and foaming properties of seven enzymatically synthesized surfactants were evaluated for the first time. Therefore, gas volume fraction, bubble size distribution and foam stability, characterized in terms of transient foam height, as well as texture were analyzed. Glycolipids consisting of different head groups, namely glucose, sorbitol, glucuronic acid and sorbose, combined with different C10 acyl chains, namely decanoate, dec-9-enoate and 4-methyl-nonanoate were compared. Equilibrium interfacial tension values varied between 24.3 and 29.6 mN/m, critical micelle concentration varied between 0.7 and 3.0 mM. In both cases highest values were found for the surfactants with unsaturated or branched tail groups. Interfacial elasticity and viscosity, however, were significantly reduced in these cases.

Head and tail group both affect foam stability. Foams from glycolipids with sorbose and glucuronic acid derived head groups were more stable than those from surfactants with glucose head group, whereas sorbitol provided lowest foam stability. This was attributed to different head group hydration which was also reflected by the time necessary to reach equilibrium interfacial adsorption. Unsaturated tail groups reduced whereas branching enhanced foam stability compared to the systems with linear, saturated tail.

Moreover, the tail group strongly influences foam texture. Glycolipids with unsaturated tail groups produced foams quickly collapsing even at smallest shear loads, whereas the branched tail group yielded a higher modulus than the linear tails. Normalized shear moduli for the systems with different head groups varied in a narrow range, with the highest value found for decylglucuronate.

Zusammenfassung

Der ökonomische Wandel weg von einer erdölbasierten Gesellschaft und Industrie hin zu einer Bioökonomie betrifft nicht nur den Energie- und Verkehrssektor, sondern alle Industriezweige und Bereiche des täglichen Lebens. So stellen auch nachhaltige Produktionswege für umweltverträgliche Tenside einen wichtigen Forschungsbereich dar. Biotenside umfassen ein breites Spektrum verschiedener Substanzen. Hierzu zählen auch Glycolipide, die sowohl chemisch als auch mikrobiell oder enzymatisch hergestellt werden können. Die enzymatische Synthese bietet den Vorteil, dass durch die Kombination unterschiedlicher Zucker(derivate) und Fettsäuren oder Fettalkoholen eine nahezu endlose Vielfalt an Glycolipiden produziert werden kann. Hierdurch wird die Herstellung von maßgeschneiderten Glycolipiden für die verschiedensten Anwendungen ermöglicht. Die Verwendung von stark eutektischen Lösungsmitteln (deep eutectic solvents, DES) als Reaktionsmedium ermöglicht eine enzymatische Glycolipid-Synthese vollständig auf Basis nachwachsender Rohstoffe.

Diese Arbeit befasst sich mit der quantitativen Analyse der enzymatischen Glycolipid-Herstellung in hydrophilen DES, woraus limitierende Faktoren identifiziert und eine Optimierungsstrategie abgeleitet wurden (Kapitel 2). Darüber hinaus wurden erstmals hydrophobe DES als Reaktionsmedien für die enzymatische Glycolipidsynthese eingesetzt und der Einfluss von verschiedenen Reaktionsparametern analysiert (Kapitel 3). In einem weiteren Schritt wurde die Struktur-Funktions-Beziehung von Glycolipiden in Bezug auf Schaumeigenschaften evaluiert, wobei Glycolipide mit unterschiedlichen Zucker(derivaten) als Kopfgruppe und Fettsäuren mit verschiedenen funktionellen Gruppen als Schwanzgruppe untersucht wurden (Kapitel 4).

Zur quantitativen Analyse der Reaktionsraten und –ausbeuten wurde in einem ersten Schritt eine HPLC-Methode mit ELS-Detektion entwickelt. Diese ermöglicht die direkte Produktquantifizierung, dadurch wird eine indirekte Analyse über den Verbrauch an Fettsäure unnötig. Die direkte Quantifizierung bietet zudem den Vorteil, dass zwischen Mono- und Di-Estern unterschieden werden kann, während dies bei der indirekten Methode nicht möglich ist. Die entwickelte HPLC-ELSD-Methode erwies sich als sehr sensitiv, da bis zu einer Glycolipid-Konzentration von 1,4 μM quantifiziert werden kann.

DES weisen im Vergleich zu Wasser und organischen Lösungsmitteln hohe Viskositäten auf. So hat das untersuchte Cholinchlorid: Harnstoff-DES (ChCl:U) bei 20 °C eine Viskosität von 0,28 Pa·s und das Cholinchlorid: Glucose-DES (ChCl:Glc) 1,41 Pa·s, während die Viskosität von Wasser bei nur 0,001 Pa·s liegt. Daher wurde ein externer Massentransferlimitierungstest durchgeführt, um zu untersuchen, ob die hohe Viskosität der DES einen limitierenden Faktor für die enzymatische Glycolipid-Synthese darstellt. Bei einer Schüttlergeschwindigkeit von mindestens 60 rpm konnte eine Massentransferlimitierung ausgeschlossen werden. Eine Fettsäurekonzentration von 0,5 mM erwies sich als geeignet für die Synthese, während höhere Fettsäurekonzentrationen zu einer Inhibierung der Lipase führten. Die mikroskopische und Laserbeugungs-Tropfengrößen-Analyse der Fettsäure-DES-Emulsionen zeigten eine bimodale Größenverteilung der Fettsäuretropfen in den hydrophilen DES-Systemen. Eine Ultraschallbehandlung der Fettsäure-DES-Emulsionen führte zu kleineren Tropfengrößen und einer monomodalen Verteilung. Durch die verbesserte Verfügbarkeit der Fettsäure für

die enzymatische Synthese konnte die Glycolipid-Ausbeute nach Ultraschallbehandlung in ChCl:U um das 4fache gesteigert werden. Somit wurde die Fettsäureverteilung als ein limitierender Faktor für die enzymatische Glycolipid-Synthese identifiziert.

Um das Problem der Fettsäureverfügbarkeit zu lösen, wurde erstmals die enzymatische Glycolipid-Synthese in einem hydrophoben DES evaluiert. Dazu wurde ein (-)-Menthol: Decansäure-DES gewählt, da dieses DES das Fettsäuresubstrat bereits enthält. Die Glycolipid-Synthese war in diesem hydrophoben DES sowohl als Veresterung mit freien Fettsäuren möglich als auch als thermodynamisch begünstigte Umesterung mit aktivierten, vinylierten Fettsäuren. Die Veresterungsreaktion hatte geringere Ausbeuten im Vergleich zur Umesterungsreaktion, da das gebildete Nebenprodukt Wasser zur Einstellung eines Reaktionsgleichgewichtes zwischen Veresterung und der Rückreaktion, einer Hydrolyse, führte. Verschiedene Reaktionsparameter wurden untersucht, um die Synthese weiter zu optimieren. 20 mg/ mL Lipase und 0,5 M Glucose ergaben die höchste anfängliche Reaktionsgeschwindigkeit für die Veresterungsreaktion, während die höchste anfängliche Reaktionsgeschwindigkeit in der Umesterungsreaktion mit 1,5 M Glucose erreicht wurde. Die Glycolipid-Ausbeuten in (-)-Menthol: Decansäure-DES waren um das 20-1000fache höher als in den untersuchten hydrophilen DES. Dieser Produktivitätsunterschied ist auf die unterschiedliche Polarität des hydrophoben DES und der hydrophilen DES zurückzuführen. Die eingesetzte Lipase war nachweislich mindestens fünf Zyklen lang ohne signifikanten Aktivitätsverlust wiederverwendbar. Somit konnten hydrophobe DES als vielversprechende Alternative zu hydrophilen DES für die Glycolipid-Synthese identifiziert werden.

Für die Auswahl von maßgeschneiderten Glycolipiden für unterschiedliche Anwendungen ist die Struktur-Funktionsbeziehung entscheidend. Daher wurden die Grenzflächeneigenschaften und die Schaumeigenschaften von sieben enzymatisch synthetisierten Glycolipiden, anhand des Gasvolumenanteil, der Blasengrößenverteilung und der Schaumstabilität, sowie der Textur erstmals analysiert. Die Schaumstabilität wurde mittels transienter Schaumhöhe charakterisiert. Hierzu wurden Glycolipide bestehend aus verschiedenen Kopfgruppen, nämlich Glucose, Sorbit, Glucuronsäure und Sorbose, kombiniert mit verschiedenen C10-Acylketten, nämlich Decanoat, Dec-9-Enoat und 4-Methyl-Nonanoat untersucht. Die Gleichgewichts-Grenzflächenspannung variierte zwischen 24,3 und 29,6 mN/m und die kritische Mizellenkonzentration zwischen 0,7 und 3,0 mM. In beiden Fällen wurden die höchsten Werte für diejenigen Glycolipide mit ungesättigten oder verzweigten Schwanzgruppen ermittelt. Grenzflächenelastizität und Viskosität waren in diesen Fällen signifikant reduziert.

Die Schaumstabilität wird sowohl durch die Kopfgruppe als auch durch die Schwanzgruppe des jeweiligen Tensides beeinflusst. Schäume aus Glycolipiden mit Sorbose- und Glucuronsäure-Kopfgruppen zeigten eine höhere Stabilität als die Schäume aus Glycolipiden mit Glucose-Kopfgruppe, wobei Sorbitol-basierte Glycolipide die niedrigste Schaumstabilität lieferten. Dies ist auf eine unterschiedliche Hydratation der Kopfgruppen zurückzuführen, welche sich auch bei den Grenzflächenspannungsmessungen in der Absorptionsgeschwindigkeit der Glycolipide an der Grenzfläche zeigte. Ungesättigte Schwanzgruppen verringerten die Schaumstabilität, während eine Verzweigung in der Alkylkette die Schaumstabilität im Vergleich zu den Systemen mit linearen, gesättigten Alkylketten erhöhte.

Darüber hinaus beeinflusst die Schwanzgruppe die Schaumtextur stark. Glycolipide mit ungesättigten Schwanzgruppen erzeugten Schäume, die selbst bei kleinsten Scherbelastungen schnell kollabierten, während die verzweigte Schwanzgruppe ein höheres Schermodul als die linearen Schwänze ergab. Die normalisierten Schermodule für die Systeme mit verschiedenen Kopfgruppen variierten in einem engen Bereich, wobei der höchste Wert für Decylglucuronat gemessen wurde.

Table of Content

1. Theoretical Background and Research Proposal

This chapter is partly based on the publication:

Parameters influencing Lipase-Catalyzed Glycolipid Synthesis by (Trans-)Esterification
Rebecca Hollenbach, Katrin Ochsenreither, Christoph Syldatk
Advances in Biochemical Engineering/Biotechnology, Springer
submitted

Authors contributions:

Rebecca Hollenbach: Conceptualization, writing original draft, review and editing of the final manuscript

Katrin Ochsenreither: conceptual advice, review and editing of the final manuscript

Christoph Syldatk: conceptual advice, supervision, review and editing of the final manuscript

Growing environmental awareness is leading to an increased demand for more sustainable strategies. The economic transition from an oil-based society and industry towards a bio-economy affects not only the energy and transport sector, but all branches of industry and areas of daily life. Therefore, the complete life cycle of products needs to be considered, from the raw material over the production process up to degradation or recycling of the product after its application. This also concerns surfactants which are applied in multimillion-ton scale per year. In this special case, renewables as building blocks for surfactants instead of petrochemicals, sustainable production processes including synthesis and solvents as well as biodegradability of surfactants need to be considered for a sustainable production of surfactants.

1.1. Surfactants

Surfactants are amphiphilic molecules consisting of a hydrophilic and a hydrophobic part. Surfactants are classified by the charge of their head group into non-ionic surfactants, anionic surfactants, cationic surfactants and amphoteric surfactants (**Table 1**)(1).

Table *1.* Surfactant classes.

Parameter	Structure	Examples
Non-ionic surfactants		Ethoxylates, alkyl polyglucosides, glycolipids
Anionic surfactants		Sulphates, sulfonates, phosphates, carboxylates
Cationic surfactants		Quaternary ammonium salts
Amphoteric surfactants		Phospholipids, phosphatidylethanolamine, phosphatidylcholine, sphingomyelins

Due to their amphiphilic character surfactants adsorb at air-liquid, liquid-liquid and solid-liquid interfaces. The adsorbed surfactants lower interfacial tension and provide a barrier against coalescence and aggregation. Therefore, surfactants are applied in the stabilization of emulsions, dispersions and foams, as well as wetting agents (2,3). As soon as the surface is covered with surfactants, micelles are formed whereby the hydrophilic head groups separate the hydrophobic tail groups from the aqueous environment. This thermodynamically-favoured self-assembly takes place when the critical micelle concentration (CMC) is reached which strongly depends on the applied surfactant, but also on temperature and ionic strength of the solution (2).

Surfactants are either petrochemically derived or bio-based. Biosurfactants are categorized into glycolipids, lipopeptides, fatty acid type biosurfactants, polymeric biosurfactants and particulate biosurfactants (4) (**Table 2**).

1.2. Glycolipids

Glycolipids are non-ionic surfactants that are not of fossil origin and can be produced entirely based on renewables. They are more ecofriendly than petrochemically-derived surfactants as they pose no risk of accumulation in the environment because they are readily biodegradable

(5–9). Moreover, glycolipids are considered as non-toxic exhibiting no mutagenic potential, low toxicity towards invertebrate and zebrafish, as well as low cytotoxicity against human epidermal keratinocytes (8,10,11).

Table 2. Biosurfactant categories.

Biosurfactants category	Examples
Glycolipids	Rhamnolipids (*Pseudomonas aeruginosa*)
Lipopeptides	Surfactin (*Bacillus subtilis*)
Fatty acid type biosurfactants	Phosphatidyl ethanolamine (*Acinetobacter* spp.)
Polymeric biosurfactants	Liposan (*Candida lipolytica*)
Particulate biosurfactants	

Glycolipids were shown to have excellent surface properties: high surface activities in combination with an efficient lowering of surface tension (12–14). They efficiently stabilize emulsions and foams (14–17). Therefore, they present a sustainable alternative to petrochemical surfactants.

Generally, surfactants have a wide field of applications in everyday life, as well as in industry. They are used in detergents, cosmetics and foods, as well as in fire-fighting and petrochemistry (3,18). Sucrose esters are glycolipids already approved for application in food industry (19). Due to their drug permeability enhancing effects glycolipids are also of relevance for the pharmaceutical industry (20). Moreover, antibacterial, anti-adhesive, antiviral and tumor inhibiting activities are reported for glycolipids (13,15,21–23).

Chemical synthesis, microbial fermentation and enzymatic synthesis are possible strategies for glycolipid production. Chemical glycolipid synthesis is industrially established on a large scale by Fischer glycosylation, which ensures low cost production with high yields (24–26). However, chemical synthesis also has a number of disadvantages: harsh reaction conditions are necessary using high temperatures and acidic catalysts (24–26). Product mixtures are generated and products are formed which make a costly purification necessary (24–26).

Rhamnolipids, sophorolipids and mannosylerithrytollipids are microbial lipids with commercial applications in cosmetic and detergent industry (18). However, structural variety of glycolipids in microbial fermentation is limited to the metabolism of the host. Low glycolipid titers in fermentation broth render purification laborious and costly (27,28).

Enzymatic synthesis is a method enabling the production of a nearly unlimited diversity of glycolipids (14,16,29,30). Thus, the tailor-made production of glycolipids gets possible. Enzymatic synthesis is based on reverse hydrolysis, which can be catalyzed enzymatically under conditions of reduced water activity (**Figure 1**). Hence organic solvents, ionic liquids (IL) and deep eutectic solvents (DES) are applicable reaction media (31–36). The use of DES

enables glycolipid production entirely based on renewables. A process solely based on lignocellulosic biomass was presented in 2018 by Siebenhaller *et al.* (37).

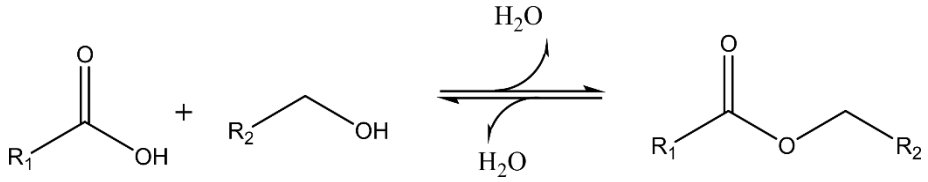

Figure 1. Reaction scheme of reversed hydrolysis.

This review discusses the latest findings on different parameters influencing enzymatic transesterification. Section 1.3. deals with deep eutectic solvents as they emerged only recently as green alternative to common solvents. Their properties and their health and environmental risk assessment will be addressed. Section 1.4. presents crucial parameters for enzymatic transesterification. Here, the role of different enzymes (Section 1.4.1.), the impact of the sugar loading (Section 1.4.3.), the influence of the fatty acid concentration (Section 1.4.4.) and the role of water in the reaction systems (Section 1.4.2.) are discussed, as well as the impact of solvent nucleophilicity and solvent hydrophobicity (Section 1.4.5.).

1.3. Deep eutectic solvents

Deep eutectic solvents were first described in 2002 by Abbott *et al.* (38). They are a mixture of two solid components, a hydrogen bond donor and a hydrogen bond acceptor, which result in a liquid at room temperature after heating or freeze-drying. DES are considered as supramolecular structures with hydrogen bond interactions (39–41). A wide range of hydrogen bond donors and acceptors are applicable for DES formation which enables tailoring of the physicochemical properties of DES (42,43). There are hydrophilic, water-miscible DES and hydrophobic, water-immiscible DES, binary and ternary DES, as well as acidic, neutral and alkaline DES covering a wide range of polarities (39,44–49). Due to this diversity, DES can be applied as "designer-solvents". DES have a high dissolution power, e.g. choline chloride: urea- and choline chloride: glycerol-DES, as well as ternary DES consisting of choline chloride or guanidine hydrochloride combined with ethylene glycol, propylene glycol or glycerol and p-toluenesulfonic acid are reported to dissolve up to 80 % of xylan and lignin from biomass (50,51). DES are reported to have stabilizing effects on enzymes while their individual components lead to enzyme denaturation. Urea leads to denaturation and inactivation of *Candida antarctica* lipase B (CalB) by disrupting hydrogen bonds of the enzyme (52). In choline chloride: urea-DES, diffusion of urea is limited due to the strong hydrogen bond network within the DES and the enzyme remains stable and active (52). The DES forms hydrogen bonds with the surface of the enzyme resulting in a more rigid structure of the enzyme and an increased thermal stability (52). In dissolutions of hydrophilic DES the supramolecular structure of DES is remained even with addition of up to 50 % water, as water gets incorporated into the hydrogen bond network, only at higher dissolution the structure of DES gets disrupted (39,40,53).

In contrast to organic liquids DES are non-volatile and non-flammable (42,43). DES have some further advantages over IL: DES are easier to prepare than ILs and due to the low cost raw materials, DES cost only about 20 % of ILs (54). Furthermore, DES have a higher biodegradability and lower toxicity compared to ILs (see chapter 3.1. and 3.2.).

The applicability of DES-buffer mixtures for fed-batch and continuous processes was shown for the enzymatic esterification of glycerol and benzoic acid in 2019 (55).

1.3.1. Toxicity of DES

DES are less cytotoxic than ILs (56). Choline chloride: amino acid DES show about 10 times lower inhibitory effects on enzymes than the imidazolium based IL [Bmim][BF4] on acetyl choline esterase and the minimal inhibitory concentration towards catalase were even 600-800 times higher than those towards acetyl choline esterase (57). DES cytotoxicity is cell line dependent and depends on the hydrogen bond donor used (56). DES with urea as hydrogen bond donor are less toxic than those with glycerol, ethylene glycol or triethylene glycol (56). Interestingly, these DES show lower cytotoxicity than aqueous solutions of their single components which indicates a reduced reactivity after DES formation due to the strong hydrogen bond network. Glucose based DES are less harmful than fructose based DES. The sugars are metabolized differently in the cells which leads to a higher formation of reactive oxygen species in fructose metabolism compared to glucose metabolism (58). The cytotoxic effects of DES are related to an increased cell membrane permeability and an increase in reactive oxygen species level (56,58).

Toxicity of hydrophobic DES has still to be assessed more thoroughly. It is merely known that menthol: lauric acid DES exhibit cytotoxicity towards HACaT cells similar to pure menthol (59).

Choline chloride: amino acid DES also showed 10-200 times lower toxicity towards bacteria than imidazolium or pyridinium derived ILs (57). DES based on choline chloride or choline acetate as hydrogen bond acceptors and acetamide, glycerol, ethylene glycol or urea as hydrogen bond donors exhibit low toxicity to bacteria at concentrations below 75 mM while they show antibacterial activity at high concentrations (60). Inhibitory effects towards gram-negative bacteria were higher than towards gram-positive bacteria, suggesting a different mode of action than conventional bacteriozides, e.g., increasing cell permeability (57,61).

Inhibitory effects of DES based on cholinium and alkanoates on growth of filamentous fungi decreased with increasing alkyl chain. The minimal inhibitory concentrations of all cholinium alkanoates were higher than those of sodium dodecyl sulfate and benzalkonium chloride (62).

Choline chloride based DES show phytotoxic effects depending on the hydrogen bond donor, while the use of ethylene glycol and acetamide shows phytotoxic effects on garlic, urea- and glycerol-DES exhibited no significant phytotoxic effect on garlic (60).

Hydras are freshwater invertebrate used for ecotoxicological studies. Choline based DES exhibit lower toxicity on hydra than their single components and therefore also represent a lower ecotoxicological burden (60,63).

1.3.2. Biodegradability of DES

Biodegradability of the solvents plays a major role in the evaluation of the environmental burden of manufacturing processes. Therefore, this is an important criterion in the selection of reaction media.

DES based on choline chloride with urea or acetamide are characterized as readily biodegradable while those with glycerol and ethylene glycol only showed biodegradability comparable to IL (60). DES based on ChCl: amino acids were also readily biodegradable (57). Likewise, the more hydrophobic DES consisting of cholinium carbonate and fatty acids showed biodegradability (62). In DES, a correlation between low toxicity and high biodegradability was observed (57). This simplifies solvent selection compared to ILs, since ILs of low toxicity usually show low biodegradability and therefore a high environmental burden (57). However, there are only a few studies existing on the biodegradability of hydrophobic DES while these data are still missing for most hydrophobic, water-immiscible DES.

1.4. Enzymatic synthesis

Success of biotransformations is strongly related to the choice of appropriate reaction conditions. Several parameters are already identified as crucial for enzymatic synthesis of glycolipids in organic solvents as well as in uncommon reaction media. Besides the selection of a suitable enzyme, the water content, substrate concentrations and solvent properties such as nucleophilicity and hydrophobicity are decisive for efficient enzymatic synthesis (**Table 3**). These parameters will be discussed in detail in the following chapter.

Table 3. Parameters positively influencing the efficiency of transesterification reactions.

Parameter	Organic solvents	Ionic liquids	Deep eutectic solvents
Sugar loading	Supersaturated solution	Supersaturated solution	Super saturated solution
Molar ratio of sugar and fatty acid	equimolar	n.e.d.	n.e.d.
Water activity	a_w<0.2	a_w~0.2	0.15<a_w<0.25
Water content	Water removal system		Addition of water up to 10 %
Solvent nucleophilicity	Low nucleophilicity	Low nucleophilicity	Low nucleophilicity
Solvent hydrophobicity		Medium polarity	
others		low halide content	

Table 3 shows which parameters were shown to work out most efficient for enzymatic glycolipid synthesis in the different solvent systems, using Novozym 435 as biocatalyst. n.e.d.: not enough data for a clear evidence.

Enzymatic glycolipid synthesis was demonstrated with three different enzyme classes: lipases, glycosidases and proteases. Glycolipid production using proteases or glycosidases was less investigated than lipase catalyzed synthesis.

Protease catalyzed synthesis of sugar fatty acid esters was successfully conducted in organic solvents using subtilisin and *Bacillus pseudofirmus* Al-89 protease (64–66). 90 % conversion was reached in a DMF/water-mixture using subtilisin (64) and 98 % conversion to sucrose laurate in 9 h using Protex 6L protease in a tert-amyl alcohol/DMSO/water solvent mixture (66). In a comparative study, Bernal *et al.* (2017) reached 57 % lactulose yield within 24 h using subtilisin and 61 % using *Thermomyces lanuginosus* lipase in acetone (67). So far, no studies on glycolipid

synthesis using proteases in DES are available. Albeit, it was shown that subtilisin exhibits transesterification activity in choline chloride: urea DES (68).

Glycosidase catalyzed synthesis of glycolipids was conducted in organic solvents and biphasic systems (69,70). Miranda-Molina *et al.* (2019) reported the first glycosidase catalyzed glycolipid synthesis in DES (71). Organic acid containing DES inactivated α-amylase within 4 h while hydrolytic activity was still measureable after 4 h in choline chloride: urea, propanedial: choline chloride: water, choline chloride: glucose: water and choline chloride: sucrose: water DES. However, at least 20 % of the co-solvent water was necessary to maintain alcoholysis activity of α-amylase (71). In choline chloride: glucose: water even 60 % water was mandatory (71). At high DES concentrations reaction rates of hydrolysis and alcoholysis reaction were decreased with hydrolysis being affected more strongly. Selectivity of methyl-glucoside synthesis was higher in DES containing reaction media than in pure buffer (71). Therefore, DES has potential for further investigations as solvent for glucosidase catalyzed glycolipid synthesis.

First lipase catalyzed lipophilization of polar substrates in DES was reported in 2013 by Durand *et al.* (72). Water activity, solvent hydrophobicity and solvent nucleophilicity are parameters that have already been identified as crucial for enzymatic glycolipid synthesis using lipases (**Table 3**).

1.4.1. Different lipases for transesterification

Several lipases have been screened for activity in DES (**Table 4**). Novozym 435 revealed to be the most effective lipase for biodiesel production in DES, followed by Lipozym TLIM while lipases from *Penicillium expansum*, *Aspergillus niger*, *Aspergillus oryzae* and *Rhizopus chinensis* showed no or only little activity (63). The study of Zhao *et al.* (2013) demonstrated that the transesterification activity of Novozym 435 in DES is also higher than that of Amano lipase, porcine pancreas lipase, *Pseudomonas cepacia* lipase and *Candida cylindracea* lipase in DES (73). Novozym 435 also proved to be a more active enzyme in the synthesis of trehalose diesters compared to Lipozym TLIM, porcine pancreas lipase and Carcia papaya lipase (17).

In a two-phase system of a IL and t-butanol Novozym 435 was the most active enzyme for glucose laurate synthesis with a conversion of 59 %, while *T. lanuginosa* lipase reached 33 % and *R. miehei* 8 % (34). *Pseudomonas cepacia* lipase, *Aspergillus sp.* acylase, *Candida antarctica* lipase A and *Candida rugosa* lipase were also tested in that system, but showed conversions of less than 5 % (34).

In organic solvents Novozym 435 was also revealed as an efficient biocatalyst. Novozym 435 showed superior performance in glycolipid synthesis in several studies compared to Lipozyme IM, *Candida antarctica* lipase A and lipases from *Rhizomucor miehei*, *Thermomyces lanuginosa*, *Pseudomonas cepacia* and *Fusarium solani* (31,74,75).

Novozym 435 was more active and stable than CalB covalently immobilized on activated silica supports, activated alumina supports, epoxy-activated sepharose and tresylated sepharose. Native CalB loses activity exponentially in a first order deactivation pattern, while Novozym 435 shows a much slower deactivation pattern (76). Due to its robustness and high activity, Novozym 435 is a promising biocatalyst for enzymatic glycolipid synthesis in DES.

Table 4. Conversions of different lipases in organic solvents, ionic liquids and deep eutectic solvents.

Solvent	Lipase	Reaction conditions	Conversion	Reference
Organic solvents	Novozym 435	Aceton, 45 °C, 72 h, Glucose palmitate, transesterification	93 %	(75)
		t-Butanol, 45 °C, 72 h, Glucose palmitate, transesterification	88 %	(75)
		2-methyl-2-butanol, 40 °C, 72 h, fructose palmitate, esterification	53 %	(31)
	Rhizomucor miehei	Aceton, 45 °C, 72 h, Glucose palmitate, transesterification	2 %	(75)
		t-Butanol, 45 °C, 72 h, Glucose palmitate, transesterification	3 %	(75)
		2-methyl-2-butanol, 40 °C, 72 h, fructose palmitate, esterification	30 %	(31)
	Thermomyces lanuginose	Aceton, 45 °C, 72 h, Glucose palmitate, transesterification	28 %	(75)
		t-Butanol, 45 °C, 72 h, Glucose palmitate, transesterification	32 %	(75)
	Pseudomonas cepacia	Aceton, 45 °C, 72 h, Glucose palmitate, transesterification	-	(75)
		t-Butanol, 45 °C, 72 h, Glucose palmitate, transesterification	3 %	(75)
Ionic liquids	Novozym 435	60 °C, 72 h, glucose fatty acid esters, transesterification [BMIM][BF4]: t-Butanol or [BMIM][PF6]: t-Butanol (3:2)	59 %	(34)
	Rhizomucor miehei		8 %	
	Thermomyces lanuginose		33 %	
	Pseudomonas cepacia		<5 %	
	Candida rugosa		<5 %	
	Candida antarctica lipase A		<5 %	
Deep eutectic solvents	Novozym 435	50 °C, 48 h, transesterification of Millettia pinnata seed oil, Choline acetate: glycerol	55 %	(63)
	Lipozym TLIM		45 %	
	Penicillium expansum		8 %	

1.4.2. Influence of water activity on lipase catalyzed transesterification

Hydration of enzymes is important for their stability and activity (77–80). However, for transesterification reaction solvents almost anhydrous conditions are necessary in order to reverse their activity from hydrolysis to esterification (81,82). Therefore, water activity is a crucial parameter in enzymatic glycolipid synthesis. Water removal systems were improving reaction yields of glucose fatty acid esters and trehalose diesters in different organic solvents with conversions up to 95 % (17,83,84).

Novozym 435 is an enzyme widely applied in transesterification reaction due to its beneficial properties. Due to the immobilization of *Candida antarctica* lipase B on a hydrophobic polymeric resin, the carriers does not strip off water from the enzyme and a sufficient hydration level is possible also at low water content of the media (76). In 2-methyl-2-butanol, highest glucose palmitate yields were reached at a water activity of 0.07, however at such low water content enzyme selectivity was reduced and the diester was produced as side product (35). Lee *et al.* (2008) reported an optimal water activity of 0.2 for transesterification reactions in ILs with Novozym 435, 0.4 with *Candida rugosa* lipase and 0.5 with Lipozym IM. At higher

water activities the reaction rates decreased (85). However, due to the strong hydrogen bond network, a defined water content is necessary for biocatalysis in DES in order to make substrates accessible. Low conversions of phenolic acids were observed without addition of water, while at 8-10 % of water (water activity between 0.15 and 0.25) almost complete transesterification occurred (72). Arabinose laurate yield in DES was significantly increased by an addition of 4 % water compared to the reaction in DES without addition of water (86).

1.4.3. Influence of sugar loading on enzymatic glycolipid synthesis

Sugar solubility is rather poor in organic solvents applied for glycolipid synthesis, such as acetonitrile, acetone, t-butanol, hexane or 2-methyl-2-butanol (83). IL and DES contain solvents with a wide range of different physical properties, so that in some, such as [Bmim][TfO] and hydrophilic DES, the sugar solubility is very good while in others it is as limited as in organic solvents (33) (37). A limited sugar solubility and thus reactant availability can strongly influence the synthesis efficiency and is therefore a crucial parameter.

Flores *et al.* (2002) showed that the dissolution of the excess sugar is not as fast as initial reaction rate in transesterification in 2-methyl-2-butanol (83). Glucose dissolution rate was enhanced by crystalline ß-Glucose and amorphous glucose resulting in higher dissolution rates and higher initial reaction rates. However, only for amorphous glucose a slightly higher yield was observed. A four times higher initial reaction rate and an 18 % higher yield was achieved by the application of supersaturated glucose solution (83). Acylation rates of disaccharides in organic solvents also depend on the dissolved sugar. Higher conversions were reported for disaccharides with a higher solubility. For the production of butanoate esters in tert-butanol yields were improved by using amorphous disaccharides compared to less soluble crystalline disaccharides (87).

Lee *et al.* (2008) could correlate enzyme activity with the dissolved sugar concentration for glycolipid synthesis in IL (33). Higher reaction rates and yields were achieved using supersaturated glucose solution than by using saturated glucose solution in IL (33). These results are in accordance to Shin *et al.* (2019) who reported higher reaction rates, yields and productivities using supersaturated sugar solutions for glucose, fructose and sucrose laurate synthesis in IL (88).

The influence of sugar loading on the enzymatic glycolipid synthesis in DES is still to be investigated.

1.4.4. Influence of fatty acid concentration on transesterification reactions

Inhibiting effects of high fatty acid concentrations were observed in transesterification reactions in organic solvents. Equimolar ratios of fatty acid and sugar led to highest yields in glucose myristate synthesis in organic solvents while fatty acid excess resulted in reduced conversions (89,90). An inhibitory effect of high fatty acid concentrations was also observed in other transesterification reactions catalyzed by *Candida antarctica* lipase B, *Candida rugosa* lipase and *Rhizopus oryzae* lipase (91–96). The inhibiting effect of fatty acids is due to the formation of non-productive complexes between fatty acids and the enzyme that are reported for reactions following ping pong mechanism (94,95,97).

Lin *et al.* (2015) reported an optimal fatty acid to sugar ratio of 1.5 for a biphasic system of ionic liquid and 2-methyl-2-butanol while productivity decreased with higher fatty acid concentrations (98). Ha *et al.* (2010) investigated sugar to fatty acid ratio from 1:1 to 1:10 in IL with highest enzyme activity for an equimolar ratio of sugar and fatty acid (99). However, Mai et al (2014) reported highest glucose laurate yields with an excess of fatty acid (sugar: fatty acid, 1:7.6) and also Galonde *et al.* (2013) reported beneficial effects of a strong excess of fatty acid on mannosyl myristate synthesis in pure IL (100). In IL with dimethyl sulfoxide as cosolvent (dimethyl sulfoxide: IL, 1:20) a sugar to fatty acid ratio of 3:1 resulted in highest conversions while at equimolar ratios or a greater excess of fatty acid yields decreased (101). The difference in these studies might be explained by the fact that Ha *et al.* used free fatty acids and supersaturated sugar solutions in an esterification while Mai *et al.* and Galonde *et al.* used vinylated fatty acids and sugar concentrations below saturation in a transesterification reaction. Therefore, the mechanism of the reaction, as well as the overall substrate loading differed between the studies which limits their comparability. During esterification reaction water is released as a side product which shifts the reaction towards hydrolysis. While in transesterification ethenol is released which tautomerizes to acetaldehyde and evaporates. Thus, the reaction gets shifted towards transesterification and is, therefore, thermodynamically favoured.

While fatty acids show in general good solubility in the organic solvents applied in transesterification, fatty acid solubility is limited in many IL (102,103). Therefore, fatty acids are not necessarily dissolved in IL, but fatty acid-solvent emulsions may be formed. This inherent difference between the solvent systems might also be an explanation for the varying observations in suitable fatty acid ratios for transesterification reaction.

Studies on influence of fatty acid concentration on glycolipid synthesis in DES are still lacking.

1.4.5. Influence of solvent hydrophobicity and nucleophilicity on lipase catalyzed transesterification

Furthermore, solvent hydrophobicity and nucleophilicity are parameters that are identified as crucial for transesterification reactions. For transesterification of 2-phenyl-1-propanol with vinylacetate, transesterification rates were higher in more hydrophobic organic solvents: methyl-t-butyl-ether > hexane > toluene > tetrahydrofuran > acetonitrile > dimethylsulfoxide (77). In organic solvents, higher sugar ester yields were achieved in less nucleophilic solvents. For transesterification using Novozym 435, Sabeder *et al.* (2006) reported higher conversions in butanone and acetone than in t-butanol (31) and Bouzaouit *et al.* (2016) reported higher reaction rates in tetrahydrofuran and butanone than t-butanol (32). t-butanol is more polar than butanone and tetrahydrofuran according to the solvatochromic parameter E_T^N (104). The same pattern was observed using *Candida antarctica* lipase B, *Mucor miehei* lipase and *Pseudomonas cepacia* lipase for lactose and sucrose ester synthesis, yields were higher in 2M2B than in acetone and lowest in methylethylketone (105). Less hydrophilic solvents have lower ability to strip off water from the enzyme (77–79).

It has also been shown for IL that the enzyme activity depends on the properties of the solvent. For transesterification of benzylalcohol with vinyl acetate, enzyme stability and enzyme activity was dependent on hydrophobicity of the ionic liquid used (85). More nucleophilic IL like [Bmin][TfO] enabled lower enzyme activity and stability than less nucleophilic, more

hydrophobic IL (85). In a transesterification study by Kaar *et al.* (2003), enzyme activity in the IL [Bmim][PF6] was higher than in organic solvents (106). However, no transesterification occurred by varying the anions resulting in more hydrophilic IL. Re-suspension of the enzyme in water revealed that inhibition was reversible with acetate and methylsulfonate anions while nitrate anions exhibited irreversible inactivation of enzymes (106). Immobilization could not enhance enzyme stability in hydrophilic IL (106). Investigations of enzyme structure using infrared spectroscopy analysis revealed a loss of the secondary structure of the enzyme in ILwith ethyl sulfate, nitrate or lactate anions (107). In these solvents transesterification activity of Novozym 435 was strongly reduced, indicating that nucleophilicity, strong hydrogen bond accepting and donating properties of IL lead to reduced lipase activity (107). Similar effects were also reported for transesterification of 2-phenyl-1-propanol with vinylacetate: transesterification rates were higher in more hydrophobic IL with higher reaction rates in [Emim][Tf2N] than in [C2OC1mim][Tf2N] and [C2OHmim][Tf2N] (77).

Ganske *et al.* (2005) reported no activity of *Candida antarctica* lipase B for synthesis of glycolipids in pure [Bmim][BF4]. However, a conversion of 59 % to glucose laurate was achieved by adding t-butanol to the ionic liquid resulting in a two phase system (34). In the less nucleophilic IL [Bmim][TfO] and [Hmim][TfO], Zhao *et al*. (2016) reported up to 26 % conversion in pure IL (108). In IL with the more nucleophilic anion methyl sulfate lower conversion was achieved even though sugars were highly soluble in that system (108). Also for those IL, higher conversion rates were achieved after mixing with an organic solvent (108). Lin *et al.* (2005) reported also that IL with methyl sulfate anion showed low conversions, while conversions in IL were better with increasing hydrophobicity of the cations (98). In a comparative study with 4 different IL and their mixtures, highest productivities combined with a high lipase stability were reported for mixtures of hydrophilic and hydrophobic IL (33).

Effects of deep eutectic solvents are less thoroughly investigated than in organic solvents or IL. However, some similarities between DES, organic solvents and IL could already be observed. Full conversion to menthyl laurate was reported for transesterification reaction using *Candida rugosa* lipase in a hydrophobic menthol: lauric acid DES (109,110).

Moreover, the anion of the hydrogen bond donor affected transesterification reactions in DES. Zhao *et al.* (2016) investigated glucose laurate synthesis in two phase systems of 2-methyl-2-butanol and DES. Almost no conversion was observed (Lipozym TLIM and Novozym 435) in choline chloride: urea and choline chloride: glycerol DES, neither with Novozym 435 nor with Lipozym TLIM, while higher conversion rates were obtained in choline acetate based DES, which were nevertheless lower than 15 % (108). Also for biodiesel production, choline acetate based DES were better suited than choline chloride based ones (63). Glycerol and ethylene glycol as hydrogen bond donor resulted in higher activity than urea or acetamide for the production of biodiesel (63). It was suggested that the hydrogen bonding network of the polyols would have an activating effect on the enzyme by interacting with a serine residue (63). Elgharbawy (2018) demonstrated increased hydrolytic lipase activity in choline chloride based DES with sugars as hydrogen bond donor for porcine pancreas lipase, Novozym 435, Immobead 150 and *Rhizopus niveus* lipase, while *Candida rugosa* lipase and Amano lipase PS stayed unaffected (111). Contrarily, malonic acid and glycerol as hydrogen bond donors showed some inhibitory effects (111). Oh *et al.* (2019) investigated lipase activity and lipase stability in various DES (44). Lipase was more active in DES with an amide hydrogen bond donor than with a polyol hydrogen bond donor, but for lipase stability the relation was

reversed (44). Still, they could not identify a correlation between solvatochromic properties of the DES and lipase activity (44).

1.5. Conclusion

The selection of the reaction conditions is a crucial step in biotransformation. For lipophilization of polar substrates, some parameters could already be identified as decisive for synthesis success independent of the solvent type.

High sugar concentrations and the use of supersaturated sugar solutions were revealed as beneficial for transesterification yields in all solvent types. In organic solvents an equimolar ratio of sugar and fatty acids resulted in highest conversion rates as an excess of fatty acids might lead to inhibitory effects. For ionic liquids and deep eutectic solvents, there are still more studies necessary to provide clear evidence as the field of applicable ionic liquids and deep eutectic solvents is a widely diverse field and solubility of fatty acids in these solvents varies considerably.

Low water activity is necessary to prevent hydrolysis of the products in organic solvents, as well as in ionic liquids and deep eutectic solvents. However, a certain water addition is mandatory in deep eutectic solvents to allow for an efficient reaction.

Solvent nucleophilicity and solvent hydrophobicity were also crucial, no matter what type of solvent was used. Selecting a solvent with low nucleophilicity promises the highest yields as no water will be stripped off from the enzyme and solvents of low nucleophilicity do not disturb enzyme structure. Nevertheless, comparative studies with solvents of different nucleophilicity and hydrophobicity are still needed, especially for deep eutectic solvents, as the currently available studies do not cover the broad spectrum of possible deep eutectic solvents systems.

1.6. Research Proposal

Glycolipids are a class of biosurfactants that present an attractive alternative to petrochemical surfactants. Their enzymatic synthesis is well studied in organic solvents but proof-of-principle studies showed that deep eutectic solvents enable enzymatic glycolipid synthesis entirely based on renewables alleviating environmental issues connected to organic solvents. Even though the applicability of deep eutectic solvents as media in biocatalysis was shown in numerous studies, there is a lack in quantitative studies on glycolipid synthesis and knowledge of parameters influencing glycolipid yields in these uncommon reaction media is missing. Furthermore, lots of highly surface-active glycolipids are known, but systematic investigations on structure-function relationship of glycolipids are still lacking.

In organic solvents, fatty acid concentration and sugar loading, as well as solvent nucleophilicity and solvent hydrophobicity were identified as decisive for synthesis success. However, little is known about the influence of these parameters on glycolipid yield in DES. DES show higher viscosities than organic solvents and their high viscosities are regarded as challenge for biocatalysis, but no research has yet been conducted on the effect of DES viscosity on mass transfer. A reliable quantification method is necessary in order to perform quantitative analysis of glycolipid synthesis. However, the existing methods use indirect quantification of glycolipids based on the consumption of fatty acids. A drawback of this quantification strategy is that monoesters and diesters cannot be distinguished.

Therefore, the aim of this thesis was to analyse the influence of different parameters:

In a first step, a direct quantification method over the glycolipid formation should be developed. Hereby, the extraction method of glycolipids from DES was also considered.

Secondly, it was intended to evaluate reaction parameters in hydrophilic DES. Two hydrophilic DES with different polarity were selected and the following parameters were investigated:

- Mass transfer by an external mass transfer limitation test
- Fatty acid concentration
- Fatty acid distribution
- Development of an optimization strategy

To further investigate the influence of different DES on glycolipid synthesis, a hydrophobic (-)-menthol: decanoic acid DES was firstly applied and the following aspects were evaluated:

- Reaction time course of esterification and side product formation (water), as well as transesterification
- Sugar loading
- Enzyme loading
- Reusability of the enzyme

A further research focus of this work was the investigation of the structure-function relationship of glycolipids. Hereby, the purpose was to produce tailor-made glycolipids with different sugar head groups and different functional groups in the fatty acid tail in order to analyse their influence on interfacial and foaming properties.

2. Optimization of Glycolipid Synthesis in Hydrophilic Deep Eutectic Solvents

This chapter is partly based on the publication:

Optimization of Glycolipid Synthesis in Hydrophilic Deep Eutectic Solvents
Rebecca Hollenbach, Benjamin Bindereif, Ulrike S. van der Schaaf, Katrin Ochsenreither, Christoph Syldatk
Frontiers in Biotechnology and Bioengineering, 2020, 8, 382.
doi: 10.3389/fbioe.2020.00382
Published: 05.05.2020

Authors contributions:

Rebecca Hollenbach: Design of the study, investigation (glycolipid synthesis, characterization of deep eutectic solvents and droplet size distribution), formal analysis, writing original draft, review and editing of the final manuscript

Benjamin Bindereif: advice for the measurement of droplet size distribution

Ulrike S. van der Schaaf: Supervision of droplet size distribution measurements,

Katrin Ochsenreither: conceptual advice, review and editing of the final manuscript

Christoph Syldatk: conceptual advice, supervision, review and editing of the final manuscript

2.1. Introduction

Glycolipids are a class of biosurfactants that have been claimed to be non-toxic (11), readily biodegradable (5,7,11), and therefore, less harmful to the environment than the petrochemically produced ones (5,8–10). Glycolipids are of special interest to the pharmaceutical industry, e.g., as bioavailability enhancers (20), and for the food industry, since e.g., sucrose fatty acid esters are approved as food additives (19,112). Apart from these applications, they can also be used in the detergent industry, textile industry and cosmetic industry, as well as in the agrochemical and the petroleum industry (3).

The enzymatic synthesis of sugar surfactants is well established in volatile organic solvents (31,113), but sugar solubility is limited in this system (83). Hydrophilic deep eutectic solvents (DES) have been reported as an alternative characterized by good sugar solubility and, in addition, non-volatility and non-flammability. DES consist of a hydrogen bond acceptor and a hydrogen bond donor (114–116). Hydrophilic DES consisting of choline as hydrogen bond acceptor and urea or glucose as hydrogen bond donor are proven to be readily biodegradable and have low cytotoxicity (58,60,117). If glucose is used as a hydrogen bond donor, it serves simultaneously as substrate for the enzymatic reaction. The synthesis of sugar surfactants in DES was first described by Pöhnlein *et al.* (2015)(36). In 2018, this process was first conducted entirely based on lignocellulosic materials (37). To date, there is only one study to be found that includes a quantitative analysis of synthesis in a DES containing system. In that study, Zhao *et al.* (2016), investigated various biphasic systems of an organic solvent with 10 % of different choline-based DES, using urea, acetamide, glycerol or ethylene glycol as the hydrogen bond donor (108). Low or negligible glycolipid yields were reported (108). Indeed, the evaluation of the limiting factors or optimization of glycolipid synthesis in DES has not been reported so far, although the high viscosity of DES is considered to be a major problem for DES applications (118), implying limited mass transfer of reactants. The investigation of different agitation rates without changing any other reaction parameter has been reported as suitable for the determination of an external mass transfer limitation (119–121). Hence, in this study, external mass transfer was investigated by using the enzymatic synthesis of glucose monodecanoate as a model reaction (**Figure 2**). In order to evaluate the influence of different reaction parameters and to identify the limitations of glycolipid synthesis in DES, and due to the challenge, posed by low concentrations on the analytics, a sensitive high performance liquid chromatography (HPLC) method with evaporative light scattering detection was developed for the analysis of glycolipids in this study. However, the high viscosity of DES reaction systems prevents a direct HPLC analysis, making sample extraction necessary. Therefore, extraction efficiency of three different extractants was also evaluated.

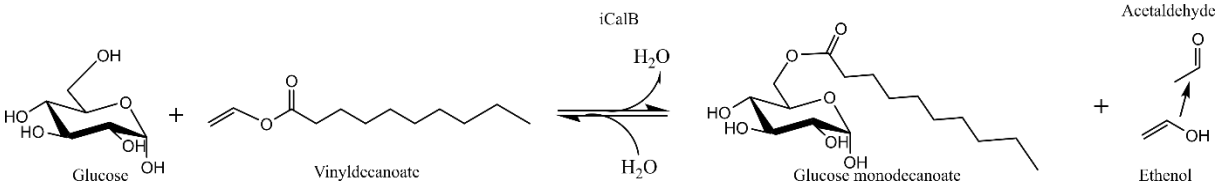

Figure 2. Reaction scheme of enzymatic glucose monodecanoate synthesis.

Two different hydrophilic DES, which were previously described in literature, were used for glycolipid synthesis. One consists of choline chloride and urea (ChCl:U), while the other contains choline chloride and glucose (ChCl:Glc). In the latter case, glucose simultaneously

contributes as part of the solvent and substrate for the reaction. Unlike organic solvents, sugar solubility is not restricted in both hydrophilic DES, but the accessibility of the second substrate, the hydrophobic fatty acid, might be limited due to the formation of fatty acid-DES emulsions. Hence, mean droplet size of the emulsion was determined as a measure for fatty acid distribution, and availability and the impact on the reaction velocity was investigated. Finally, with the results obtained, a strategy to optimize the reaction can be developed.

2.2. Materials and Methods

2.2.1. Materials

All chemicals were acquired from Carl-Roth (Germany) if not stated otherwise. All solvents were in HPLC grade. Lipase B from *Candida antarctica*, immobilized on acrylic resin (iCalB), was purchased from Strem Chemicals (Strem chemicals Europe, Germany). Vinyl decanoic acid was acquired from Tokyo Chemical Industry Co., Ltd. (TCIEurope, Belgium). 6-Decanoyl-D-glucose was purchased from Sohena (Germany). Double distilled water (0.005 mS) was obtained using a Purelab flex water system from Elga LabWater (Celle, Germany).

2.2.2. Preparation of DES

Two different DES based on choline chloride were used in this study: choline chloride urea (ChCl:U) and choline chloride glucose (ChCl:Glc). For the preparation of ChCl:U, choline chloride and urea were mixed in a molar ratio of 1:2 (n:n) and 5 % (v/v) of double distilled water was added. For ChCl:Glc, choline chloride, glucose and water were mixed in a ratio of 5:2:5 (n:n:n).

The mixtures (200 g for each DES) were heated in a sealed glass bottle to a temperature of 90 °C and stirred at 600 rpm using a NeoMag magnetic stirrer from neoLab (Heidelberg, Germany) for 2 h until a colorless fluid was obtained. Then, the DES were allowed to cool to room temperature.

2.2.3. Ultrasonic Pretreatment

Samples of 10 mL DES containing 0.5 M vinyldecanoate were sonicated for 5 min with 60 % amplitude and a cycle of 20 s pulsing and 30 s pause in 50 mL tubes. A Sonopuls HD 3100 ultrasonic homogenizer from Bandelin (Berlin, Germany) equipped with a MS 72 probe was used at a frequency of 20 kHz (with an energy input of 4.654 kJ). The probe was set to an immersion depth of 1.5 cm. During sonication, the samples were cooled in a water bath after which they were immediately used for synthesis.

2.2.4. Synthesis of Glycolipids (Standard Reaction)

Glycolipid synthesis was carried out in 5 mL tubes (Eppendorf AG, Hamburg, Germany) filled with 2 mL DES. Vinyl decanoate was added to a final concentration of 0.5 M to both DES, while glucose (final concentration 0.5 M) was added only to ChCl:U. Finally, by adding 20 mg/mL iCalB the reaction was started. The tubes containing 2.5 g of reaction mixture, were mixed in a rotator with a vortex mixer (program U2) from neoLab (Heidelberg, Germany) at working conditions of 90 rpm and 50 °C. The reaction time varied between 4 h (to determine the initial

reaction velocity) and 24 h (to calculate the total yield). At each time point of interest, three tubes (for triplicate measure) were collected and totally processed for further analysis.

2.2.5. External Mass Transfer Limitation Test

In order to examine external mass transfer limitation, the agitation rates were varied from 30 rpm to 60 rpm and 90 rpm with a reaction time of 4 h. All other reaction conditions were kept constant.

2.2.6. Influence of Fatty Acid Concentration

To address the optimal fatty acid concentration for the reaction, different fatty acid concentrations (0.25, 0.5, 0.75, and 1.0 M) were tested without varying any other reaction parameter.

2.2.7. Synthesis With Ultrasonic Pretreatment

For the synthesis with ultrasonic pretreatment, 2 mL of each sonicated fatty acid-DES emulsion was filled into 5 mL tubes. In the case of ChCl:U, glucose (0.5 M) was supplemented. Finally, 20 mg/mL iCalB was added. The other reaction conditions were remained same as the standard reaction.

2.2.8. Extraction of Glycolipids

For glycolipid extraction, 3.42 mL of extraction solvent and 1.1 mL of double distilled water were mixed with the reaction tube content (2.5 g) in a rotator with vortex mixer (program U2) at an agitation rate of 90 rpm. Temperature and time were set to 50 °C and 20 min, respectively, after which the upper organic phase was collected for HPLC analysis.

Three different extraction solvents were tested under similar conditions as stated above, but the volume was halved (1.71 mL of extraction solvent and 0.55 mL of double distilled water per mL of DES): ethyl acetate (EtAc), dimethyl carbonate (DMC) and chloroform were tested as extractants. To evaluate the extraction performances, 5 mg of glucose decanoate was incubated in 1 mL of both DES for at least 5 h and afterward extracted and analyzed by HPLC-ELSD. The extraction efficiency was calculated as follows:

$$\text{extraction efficiency } [\%] = \frac{\text{glucose monodecanoate content measured}}{\text{glucose monodecanoate content added}} \times 100\ \%$$

2.2.9. HPLC-Evaporative Light Scattering Detector (ELSD)

Glucose decanoate was determined by HPLC using a Kinetex EVO C18 (2.6 mm, 250 mm × 4.6 mm) column from Phenomenex (Aschaffenburg, Germany) with an accompanying guard column (4 mm × 3.0 mm ID) of the same phase, using an Agilent 1260 series liquid chromatograph (Waldbronn, Germany) equipped with a quaternary pump, an autosampler and a column oven. An evaporative light scattering detector (ELSD) from BÜCHI Labortechnik (Essen, Germany) was used for detection. The mobile phase, solvent A, was water and solvent B was acetonitrile. The flow rate was 1 mL/min and a gradient was used for separation of products and substrates: starting from 40 % A-60 % B, then 0–10 min 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. The injection volume was set to 10 mL. The column was operated at 50 °C. The detector was operated at 38 °C with a gas flow (air) of 1.5 mL/min. The gain was set to 1.

2.2.10. Viscosity Measurements

The viscosity of DES was measured using a Physica MCR 101 viscometer (Anton Paar, Ostfildern, Germany) with double gap geometry (DG26.7) and shear rates of 2-100 s^{-1}. Measurements were conducted at temperatures of 20 °C and 50 °C.

2.2.11. Droplet Size Distribution Measurements

The droplet size distribution of vinyl decanoate in DES emulsions was characterized using a Horiba LA-940 laser diffraction particle analyzer from Retsch Technology GmbH (Haan, Germany). Refractive indices required for the calculation of the droplet size distribution by the built-in software were determined as 1.4362 for vinyl decanoate, 1.4971 for ChCl:U and 1.4981 for ChCl:Glc by an analogous Abbe refractometer AR4 (Krüss Optronic, Hamburg, Germany). Samples were measured directly after preparation.

2.2.12. Statistical Analysis

Results are presented as mean ± standard deviation (n = 3). Statistical data analysis was performed by two-way ANOVA and Tukey test using the software OriginPro 9.6 (version 2019; OriginLab, Nothampton, Massachusetts, USA). Results were considered significant if p-value was <0.05.

2.3. Results

The main purpose of this study was to identify the limiting factors and therefore optimization potential for glycolipid synthesis in two hydrophilic DES requiring a reliable and sensitive quantification method. The first part therefore describes the development and evaluation of a glycolipid extraction method, as well as the quantification of the model substrate glucose monodecanote by HPLC-ELSD.

2.3.1. Quantification of Glucose Monodecanoate

Glucose monodecanoate was successfully separated from glucose and vinyl decanoate using the developed HPLC-ELSD method (**Appendix: Figure A1**). The retention times were 2.1 min for glucose, 2.68 min for glucose monodecanoate and 5.76 min for vinyl decanoate. Due to the low baseline noise and the peak resolution (**Table 5**), glucose monodecanoate can be quantified in a range between 0.0014 µmol/ml and 4.49 µmol/ml.

Table 5. Chromatographic and analytical characteristics of glucose monodecanoate analysis using HPLC-ELSD

Analytical characteristics	
Retention time (glucose monodedcanoate)[a]	2.68 min – 2.72 min
Correlation coefficient (R2, n=3)	0.9975
Equation of linear calibration	y=9759.9x-247.87
Linear range of calibration	0.06 - 4.49 µmol/mL
Resolution glucose - glucose monodecanoate (n=3)	7.7
Resolution glucose monodecanoate – decanoic acid (n=3)	29.5
Peak width[b]	0.039 – 0.054 min
Baseline noise (n=3)	0.06 mV
Limit of detection (signal/noise=3)	<0.0014 µmol/mL
Limit of quantification (signal/noise=10)	<0.0014 µmol/mL

[a] inter-day variance of retention time measured at 3 different days

[b] concentration 0.06 – 4.49 µmol/mL

2.3.2. Extraction Efficiency of Different Solvents

In order to provide a reliable extraction method for the quantification of glycolipids, three different extraction solvents were tested (**Figure 3**). In this regard, chloroform, EtAc and DMC were used to extract glucose decanoate from the DES, and their performance was evaluated. With all three solvents, a two-phase system was formed: a DES-water phase and an organic solvent phase containing the glucose monodecanoate. Chloroform was the worst extraction solvent with an efficiency of a single extraction of 30.6 ± 2.5 % (ChCl:U) and 27.3 ± 9.6 % (ChCl:Glc). In contrast, the solvents EtAc and DMC were statistically more effective with EtAc showing the highest yields of 81.4 ± 2.5 % (ChCl:U) and 94.4 ± 13.3 % (ChCl:Glc). Therefore, EtAc was chosen as an extractant for this study.

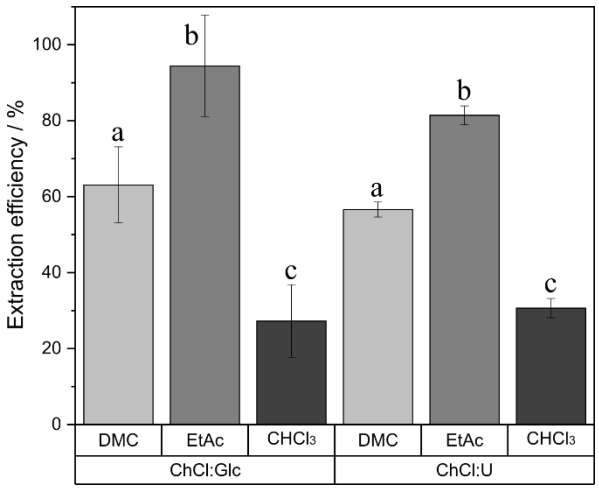

Figure 3. Comparison of three extraction solvents, dimethyl carbonate (DMC), ethyl acetate (EtAc) and chloroform ($CHCl_3$), for glucose monodecanoate extraction from ChCl:Glc (ChCl:Glc:water, 5:2:5, n:n:n) and ChCl:U (ChCl:U, 1:2, n:n, 5 % water). a, b, c show statistically significant differences.

2.3.3. External Mass Transfer Limitation Test

In order to investigate the effect of viscosity on glycolipid yield, the viscosity of both types of DES was measured. Both DES showed Newtonian behavior, with ChCl:U having a viscosity of 0.28 ± 0.03 Pa·s and ChCl:Glc of 1.41 ± 0.16 Pa·s at 20 °C. For contrast, the viscosity of water

is 0.001 ± 0.00 Pa·s. At 50 °C, the viscosity of ChCl:U is 0.053 ± 0.0004 Pa·s and that of ChCl:Glc is 0.17 ± 0.002 Pa·s.

To examine the effect of the high viscosity of DES on external mass transfer, an experiment with different agitation rates (30, 60, and 90 rpm) was set up and the initial reaction velocity was analyzed (**Figure 4**). There was neither a statistical difference concerning the initial reaction velocity between the two investigated DES nor a significant interaction between the type of DES and the agitation rate. For both DES, there was significant increase in the initial reaction velocity with increasing agitation rate. However, higher agitation rates than 60 rpm had no effect on the reaction rates.

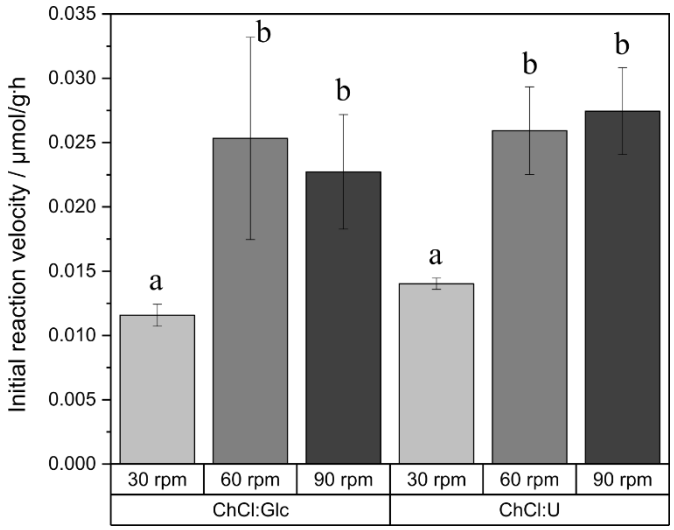

Figure 4. Initial reaction velocity in relation to the agitation rate. Glucose monodecanoate was determined directly by product quantification. Reaction conditions: 0.5 M vinyl decanoic acid, 50 °C. a, b show statistically significant differences. ChCl:Glc (ChCl:Glc:water, 5:2:5, n:n:n) and ChCl:U (ChCl:U, 1:2, n:n, 5 % water).

2.3.4. Influence of Fatty Acid Concentration

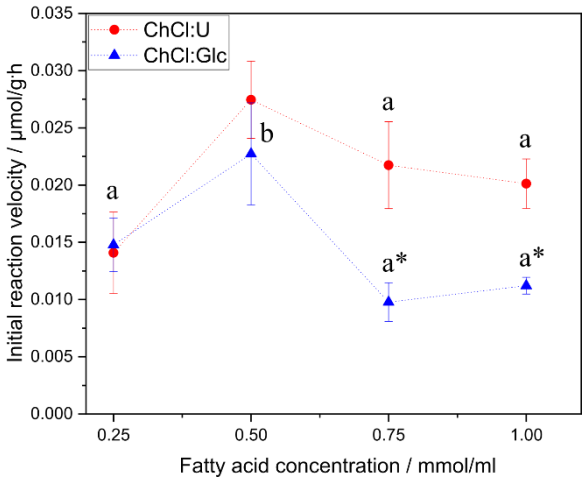

Figure 5. Impact of different fatty acid concentrations on the initial reaction velocity in ChCl:U (ChCl:U, 1:2, n:n, 5 % water) and ChCl:Glc (ChCl:Glc:water, 5:2:5, n:n:n). Glucose monodecanoate was determined directly by product quantification. Reaction conditions: 90 rpm, 50 °C. a, b show statistically significant differences between fatty acid concentrations. * shows statistically significant differences between the two DES.

Investigations of the initial reaction velocity in relation to the fatty acid concentration revealed a significant increase in the initial reaction velocity, with an increase in fatty acid concentration from 0.25 to 0.5 mol/L (**Figure 5**). However, a further increase in the fatty acid concentration did not cause any increase in the initial reaction velocity; rather, it resulted in a reduced initial reaction rate at fatty acid concentrations higher than 0.5 mol/L for both DES.

2.3.5. Influence of Fatty Acid Distribution

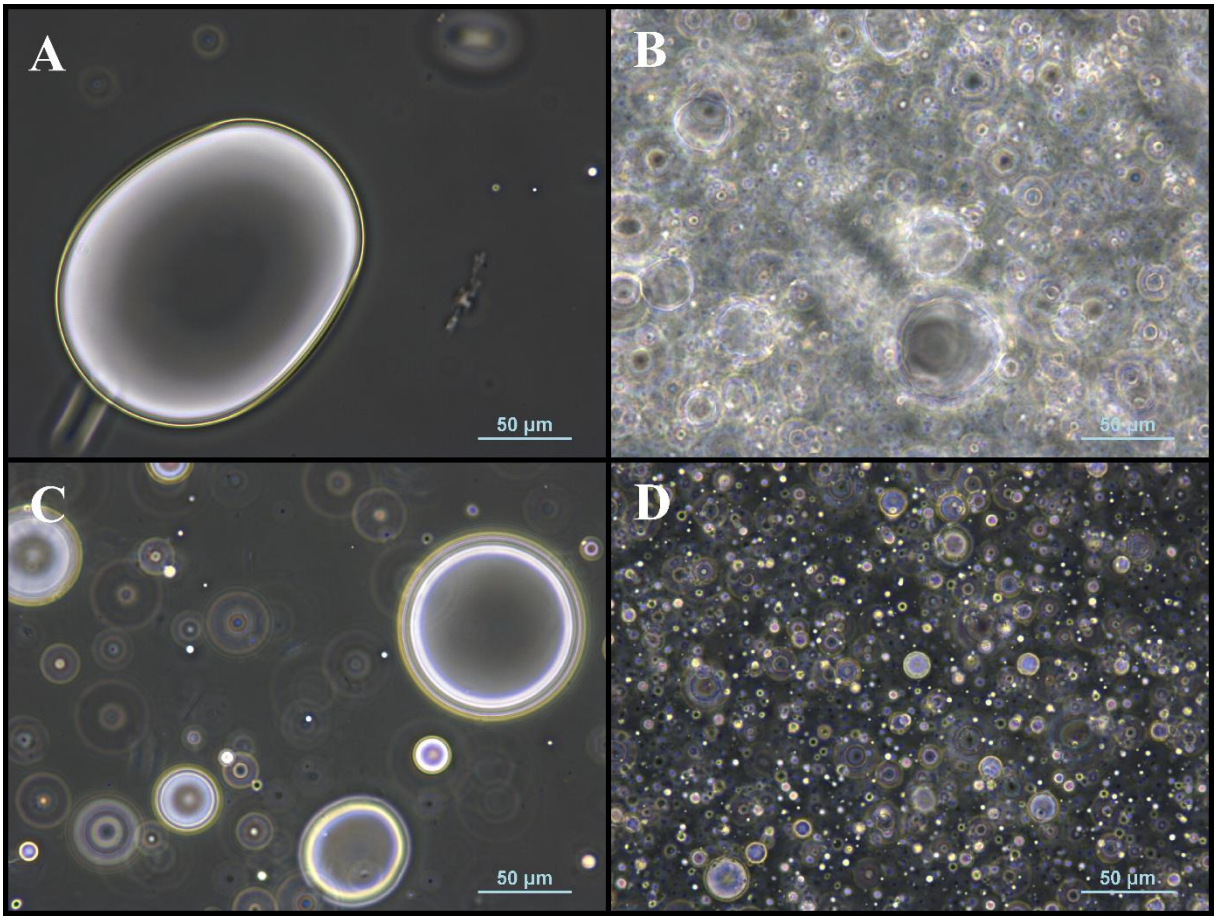

Figure 6. Microscopic pictures of untreated and sonicated fatty acid-DES emulsions. A shows untreated fatty acid-ChCl:Glc emulsion, B sonicated fatty acid-ChCl:Glc emulsion, C untreated fatty acid-ChCl:U emulsion and D sonicated fatty acid-ChCl:U emulsion. Fatty acid concentration in all fatty acid-DES emulsions was 0.5 M. The images were obtained using phase contrast and a Nikon Eclipse E200 microscope.

The effect of an ultrasonic treatment on fatty acid distribution in fatty acid-DES emulsions and on the resulting reaction rates was investigated. Optical microscopic analysis of fatty acid-DES emulsions showed smaller and more homogenously distributed fatty acid droplets after sonication for both DES. The droplet sizes obtained were smaller for ChCl:U than for ChCl:Glc (**Figure 6**). The untreated fatty acid-DES emulsions showed bimodality (**Figure 7**). The cumulative volume distribution of fatty acid-DES emulsions shifted toward smaller droplet diameters after sonication treatment, and bimodality was reduced. Significant differences in the mean droplet size $x_{50;3}$ were determined between the two investigated DES as well as between untreated and sonicated DES. There was reduction in the mean droplet size $x_{50;3}$ by sonication for both DES: for ChCl:U, from 54 ± 7 µm to 35 ± 2 µm, and for ChCl:Glc, from 464 ± 250 µm to 51 ± 13 µm.

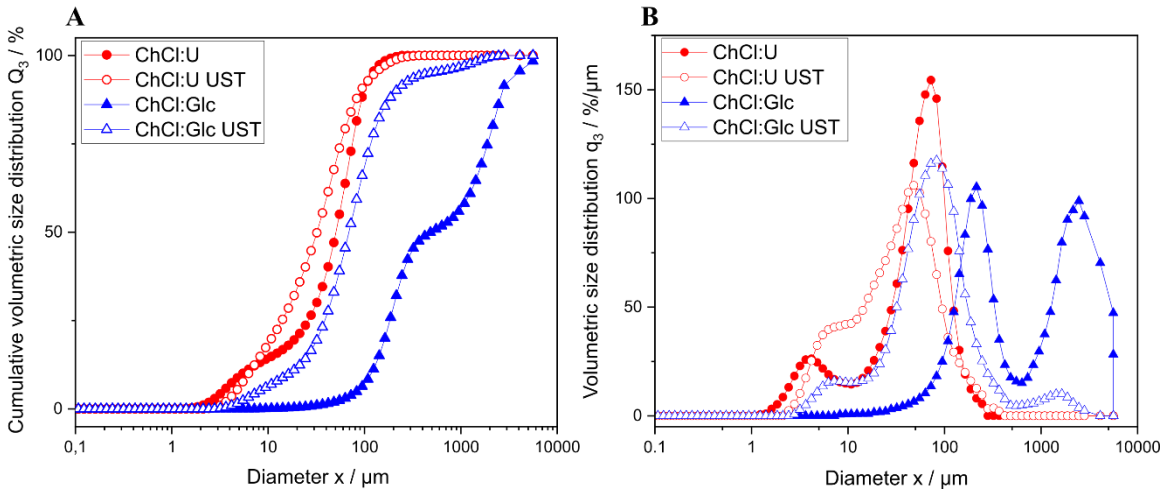

Figure 7. Impact of ultrasonic pretreatment (UST) on drople size distribution of fatty acid-DES emulsions. A shows the cumulative volumetric size distribution Q_3 and B the volumetric size distribution q_3.

Statistical analysis of initial reaction velocity in relation to ultrasonic pretreatment revealed a significant difference between ChCl:U and ChCl:Glc (**Figure 8**). The ultrasonic pretreatment had an influence on the initial reaction velocity but only for ChCl:U. The initial reaction rate of ChCl:U was significantly accelerated from 0.026 ± 0.003 µmol glucose monodecanoate/g DES·h to 0.056 ± 0.014 µmol/g·h. The glycolipid yield after 24 h synthesis in ChCl:U was increased by ultrasonic pretreatment from 0.15 ± 0.029 µmol/g to 0.57 ± 0.029 µmol/g.

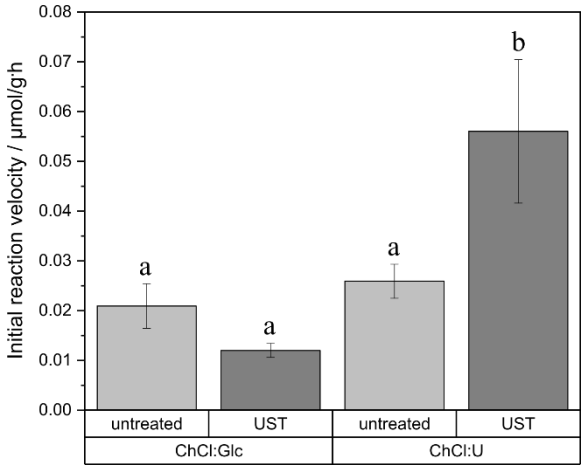

Figure 8. Impact of ultrasonic pretreatment (UST) on initial reaction velocity in ChCl:Glc (ChCl:Glc:water, 5:2:5, n:n:n) and ChCl:U (ChCl:U, 1:2, n:n, 5 % water). Glucose monodecanoate was determined directly by product quantification. Reaction conditions: 0.5 M vinyl decanoic acid, 50 °C, 90 rpm. a, b show statistically significant differences.

2.4. Discussion

The developed direct product quantification method enables the separation and quantification of monoesters and multiple esterified products, as well as educts which is more advantageous than the indirect analysis via substrate consumption. In the studies by Šabeder *et al.* (2006) and Bouzaouit and Bidjou-haiour (2016), an indirect quantification via the fatty acid amount, was

performed (31,32). However, that quantification method can lead to an overestimation of the actual monoester formation, as substrate consumption offers no differentiation between monoesters and multiple esterified products. In contrast, direct quantification enables the identification and monitoring of different reaction phases, i.e., the transition from formation of monoesters to di- or polyesters. In the studies where a direct glycolipid quantification via HPLC was performed, refractive index detection was used (98,108,113). However, refractive index detection is incompatible with gradient elution, but ELSD detection allows for this (122,123). In addition, ELSD detection has a higher sensitivity compared to refractive index detection, thus more suitable for low product concentrations (122–124).

Due to the viscosity of the investigated DES, it was not possible to directly inject the samples into the HPLC, so that either a dilution step must be performed to reduce the viscosity of the sample, as proposed by Zhao *et al.* (2016), or sample extraction must be performed. In the case of ChCl:Glc, an extraction is mandatory in order to avoid overloading the column with glucose and the associated poor separation of glucose and glucose decanoate. In addition, a strong dilution may conceal low concentration products. Since concentrations of 0.5 mmol/mL and lower were expected on the basis of preliminary studies and the findings of Zhao *et al.* (2016), a sample extraction approach was chosen in this study to overcome that issue.

To the best of our knowledge, there are no studies evaluating the extraction solvents for glycolipid extraction from DES. In the eighties, chloroform was still used as solvent for biosurfactant extraction from fermentation broth (125,126). Later, EtAc started replacing chloroform as an extraction solvent (29,127). More recently, to extract polar and nonpolar lipids from microalgae, DMC is used as an alternative to traditional chloroform extraction (128,129). On this account, these three solvents have been chosen to be investigated. The more similar a certain solvent and the compound of interest, the better the solubility of that particular solvent is. To quantitatively evaluate this, and interpret the observed results, solubility parameters, e.g., Hansen solubility parameters, are a useful tool. Hansen solubility parameters describe solvent properties like nonpolar interaction, dipolar interaction and hydrogen bonding interaction (130). Hansen parameters for chloroform indicate increased nonpolar interactions than ethyl acetate and DMC, as well as lower dipolar and hydrogen bonding interactions (131). Thus, the theoretical and experimental results coincide, since glycolipids are polar molecules and chloroform presents the worst results of the three solvents investigated. DMC has higher Hansen parameters for dipolar and hydrogen bonding interactions than EtAc (131). As DMC is less efficient in glucose monodecanoate extraction than EtAc, it is concluded that the amphiphilic glucose monodecanoate is less polar than DMC and therefore better extractable with less polar EtAc.

Furthermore, safety and toxicology are important aspects when selecting an extraction solvent. For the classification of solvents as green solvents, the following aspects must be considered: the entire life cycle of the solvents, safety in handling, health hazards and environmental compatibility (132,133). EtAc and DMC are classified as recommended solvents while chloroform is classified as highly hazardous and should be avoided even in the laboratory (133). The investigation of commonly applied chloroform, EtAc, and uncommon DMC as solvents for glycolipid extraction from DES showed that the green solvents EtAc and DMC have a statistically higher efficiency than the harmful commonly applied chloroform. Based on extrapolation from the yield of sonicated ChCl:U experiments, 894 mL EtAc, 1307 mL DMC or 2429 mL $CHCl_3$ would be necessary for the extraction of 1 g glucose monodecanoate.

Therefore, it is advisable to use EtAc, which was confirmed as the most efficient extraction solvent, or DMC for glycolipid extraction.

The measured viscosity of ChCl:Glc is in good accordance with the value reported by Dai *et al.* (2013)(118). Viscosity of DES are 100 to 2000 times higher than those of organic solvents (134,135), and are therefore regarded as the major problem of DES applications since they may pose mass transfer limitations (108,118). Instead, the results of the external mass transfer limitation test show that, at least in our process, viscosity is only a limiting factor for glycolipid synthesis at agitation rates below 60 rpm. At agitation rates of 60 rpm and higher, external mass transfer limitation due to the high viscosity of DES can be excluded, since the statistical analysis revealed no difference between the two differently viscous DES and there is no statistical difference in reaction velocities. These findings also apply for sonicated samples (**Appendix: Figure A2**). Therefore, it can be assumed that the mixing is sufficient and there is no external mass transfer limitation for non-sonicated samples, as well as for sonicated samples. Similar results with regard to external mass transfer in DES were described by Ülger and Takaç (2017), where methyl gallate synthesis in DES was not enhanced at higher agitation rates after a certain threshold (121). Moreover, the relationship between agitation rate and initial reaction velocity was also used as a measure of external mass transfer in organic media and in enzymatic synthesis of antibiotics (119,120).

Investigations of the influence of fatty acid concentration on the initial reaction velocity revealed an inhibiting effect of fatty acid concentrations higher than 0.5 mol/L. Similar findings were reported for glucosyl myristate synthesis in organic solvents (90,136). Besides, fatty acid inhibition of CalB was also reported for other transesterification reactions, e.g., fatty acid esters, acetoin fatty acid esters or citronellol laurate (92,97,137). Esterification and transesterification reactions follow a ping pong mechanism, and fatty acids are inhibitors by forming non-productive complexes with the enzyme (95,97,138). This effect of fatty acids on esterification reactions was also observed for other lipases than CalB, e.g., Candida rugosa lipase or Rhizopus oryzae lipase (95,96,138).

Once the existence of an external mass transfer limitation due to the high viscosities of DES could be excluded and an appropriate fatty acid concentration was chosen, it was investigated whether the fatty acid accessibility is a limiting factor. Sonication was reported as an effective method for emulsification (139) and was therefore selected as treatment for improving fatty acid distribution. Pandolfe (1981) reported a decrease in efficiency of sonication with increasing viscosity of the continuous phase; though at high viscosities (>0.1 Pa·s) this is no longer true (140). However, we observed significantly smaller mean droplet sizes for both the less and the more viscous DES upon sonication. Nevertheless, the resulting droplet size upon sonication was smaller for ChCl:U, the less viscous DES, than for ChCl:Glc. Remarkably, for the more viscous DES ChCl:Glc a considerably greater droplet size reduction was achieved (by 89 %) compared to the less viscous ChCl:U (by 36 %).

Ultrasonic pretreatment led to a statistically significant higher initial reaction velocity in ChCl:U, as well as to an improved overall yield, and to a cumulative volume size distribution, which shifted toward smaller fatty acid droplet size. Hence, fatty acid distribution can be assumed as a limiting factor. Despite the stark droplet size reduction in ChCl:Glc, the initial reaction rate did not increase significantly upon sonication. In contrast, for ChCl:U the initial reaction rate increased significantly upon sonication although the mean droplet size decreased

only slightly. Therefore, the different performances of the two tested fatty acid-DES mixtures might more likely be the result of the differences in polarity of the DES than due to their different viscosities: according to the solvatochromic parameter E_T^N, ChCl:U (0.835) is less polar than ChCl:Glc (0.845) (44). Oh *et al.* (2019) also showed that lipase activity in the transesterification of benzylalcohol and vinylacetate is higher in DES containing urea as hydrogen bond donor than in more polar DES containing glucose as hydrogen bond donor (44). An influence of solvent polarity on transesterification reactions was also reported for organic solvents and ionic liquids (IL). In organic solvents, the reaction yield in glycolipid synthesis with butanone as solvent is higher than with the more polar solvents acetone and t-butanol (31,32). Various IL investigations have shown that a compromise between highly hydrophilic ILs for good sugar solubility and highly hydrophobic IL for good fatty acid solubility is needed to achieve good conversion rates (98). Thus, both literature and the reported experimental results show that the polarity of the solvent is another factor that plays a role in glycolipid synthesis in DES.

Besides the fatty acid, the alcohol substrate is also reported to act as an inhibitor on enzyme activity in esterification reactions by forming dead-end complexes (97,138). Therefore the glucose excess in ChCl:Glc might also contribute to the lower reaction yields compared to ChCl:U.

Also, other factors might be contributing to the different performances of the two DES, such as the different strength and nature of the hydrogen bonding network in the DES. The literature indicates that DES may contribute to lipase stability and activity by forming hydrogen bonds between DES and lipase, thereby stabilizing the tertiary structure of the enzyme (44,111,141). On the other hand, DES may also lower the lipase activity by destabilizing enzymesubstrate complexes or intermediate complexes (44,111,141). The stabilizing and destabilizing effects of hydrogen bonding between DES and lipase are in correlation with the nature of the DES, hydrogen bond acidity, and hydrogen bond basicity (44,141).

2.5. Conclusion

In this study, limiting factors of glycolipid synthesis in DES were addressed and an optimization strategy was presented. For this, a quantification method consisting of an extraction method and an HPLC-ELSD measurement was developed, an external mass transfer limitation test was applied, the fatty acid concentration was optimized and the influence of droplet size distribution in fatty acid-DES emulsions on initial reaction velocities and glycolipid yield were investigated.

No differences in initial reaction velocities were observed for agitation rates of 60 rpm and higher. Therefore, it was shown that by using a proper agitation rate, an external mass transfer limitation in the investigated DES can be excluded. Results of the droplet size distribution measurements and the study of the initial reaction velocity of sonicated DES-fatty acid emulsions revealed that the fatty acid distribution is a limiting factor for glycolipid synthesis in ChCl:U. By applying a sonication treatment, the glucose decanoate yield of the enzymatic synthesis in ChCl:U was increased fourfold. However, despite obtaining a droplet size reduction, the initial reaction velocity of the more polar DES, ChCl:Glc, was not increased upon sonication. The effect of the polarity of DES on enzymatic glycolipid production should

therefore be further addressed in a following study. In addition, alternative processes to sonication should be investigated as well as the use of adjuvants, both with the aim of reducing droplet size distributions. This will make the process more efficient and economical and will eventually make it possible to scale up.

3. Enzymatic Glucose monodecanoate Synthesis in a Hydrophobic Deep Eutectic Solvent

This chapter is partly based on the publication:

Enzymatic Synthesis of Glucose Monodecanoate in a Hydrophobic Deep Eutectic Solvent
Rebecca Hollenbach, Katrin Ochsenreither, Christoph Syldatk
International Journal of Molecular Science, 2020, 21, 4342.
doi: 10.3390/ijms21124342
Published: 18.06.2020

Authors Contribution:

Rebecca Hollenbach: Design of the study, investigation, formal analysis, writing original draft, review and editing of the final manuscript

Katrin Ochsenreither: conceptual advice, review and editing of the final manuscript

Christoph Syldatk: conceptual advice, supervision, review and editing of the final manuscript

3.1. Introduction

Surfactants are amphiphilic molecules that are applied in numerous industries and in personal care on a daily basis and a multimillion-ton scale per year (3,4,19,20). The majority of surfactants are still based on fossil resources; however, due to environmental awareness there are also more ecofriendly alternatives, i.e., biosurfactants produced from renewables are getting more attention. Glycolipids, consisting of a sugar moiety acylated with an alkyl chain, are the biggest group within the biosurfactants. They are characterized by properties similar or even superior to their petrochemical counterparts while being biodegradable, non-toxic and skin-friendly (7–9).

Glycolipids can be synthesized by microbial fermentation, e.g., sophorose lipids and rhamnolipids, and by chemical or enzymatic synthesis (29,142). While microbial fermentation is limited to certain molecules, chemical and enzymatical synthesis can be used for tailor-made glycolipid synthesis with theoretically no restriction regarding sugar moiety and alkyl chain. Enzymatic synthesis additionally offers stereo- and regio-selectivity. For the linkage of saccharide and alkyl chain, a reaction solvent of low water activity is needed as reversed hydrolysis only occurs under conditions of reduced water activity (35,76,81). Organic solvents are frequently used; however, the suitability of ionic liquids (IL) and deep eutectic solvents (DES) have also been studied. In contrast to organic solvents, DES and IL are non-volatile and non-flammable (38,143). Furthermore, DES are reported to be biodegradable and non-toxic (58,117,144) while exhibiting a high dissolution power for many different materials, including drugs, proteins, salts, sugars and surfactants (145). DES are applicable as solvent or catalyst in a wide range of organic reactions, e.g., addition, cyclization, condensation and multicomponent reactions, and improve the activity or selectivity of these reactions compared to organic solvents (146–148). However, DES present a suitable solvent not only for organic reactions but also for biocatalysis. Lipases, glucosidases and proteases are among the enzymes that have been successfully used in DES to catalyze, for example, (trans-)esterification, Aldol and Henry reactions, as well as deglycosylation, dehalogenation, epoxide hydrolysis and oxidation reactions (143). Initial reaction velocities as well as enzyme stability differ in various DES due to the varying hydrogen bond network depending on their constituents (145). At the same time, a strong hydrogen bond network between DES and reaction substrates can lead to a limited availability of those for the reaction (72). Therefore, it is necessary to select a DES suitable for substrates and enzymes. In some cases, a DES serves as solvent and as substrate for the enzymatic reaction (29).

However, the knowledge of glycolipid synthesis in DES is still limited. So far, only hydrophilic DES were used for glycolipid synthesis (29,103,149), although literature indicates that solvent polarity might have an impact on glycolipid synthesis. Enzyme stability is reduced in polar solvents due to stripping off hydration water from the enzyme (78). Solvents of medium polarity present a compromise between enzyme stability and sugar solubility and showed, therefore, highest yields in organic solvents as well as in IL (31,32,98). Furthermore, fatty acid availability is a limiting factor of glycolipid synthesis in hydrophilic DES (103).

Thus, the aim of this study was to investigate whether enzymatic synthesis of glycolipids is possible not only in hydrophilic DESs but also in a hydrophobic DES. For this purpose, a hydrophobic DES consisting of (-)-menthol and decanoic acid was used for the synthesis of glucose monodecanoate for the first time. Hydrophobic DES are a new class of solvents that

were described in 2015 for the first time (46). (-)-menthol: decanoic acid DES was chosen as this DES can serve as solvent and substrate simultaneously, and low water solubility was reported, which is beneficial for reversed hydrolysis reactions (45,47). Furthermore, this DES was recently described to be suitable for an enzymatic reaction using *Candida rugosa* Lipase (109).

The glycolipid syntheses conducted in DESs have used mostly vinylated fatty acids as substrates, but no quantitative studies using free fatty acids in DESs have been conducted yet. However, the transesterification reaction introduces an additional reaction step as the fatty acids have to be vinylated prior to the reaction. Thus, the esterification reaction is preferred in terms of green chemistry. Therefore, esterification as well as transesterification were considered and compared in this study (**Figure 9**). Furthermore, the impact of the hydrophobic DESs on reaction rates was evaluated and several reaction parameters were examined in order to characterize the reaction in this novel reaction medium.

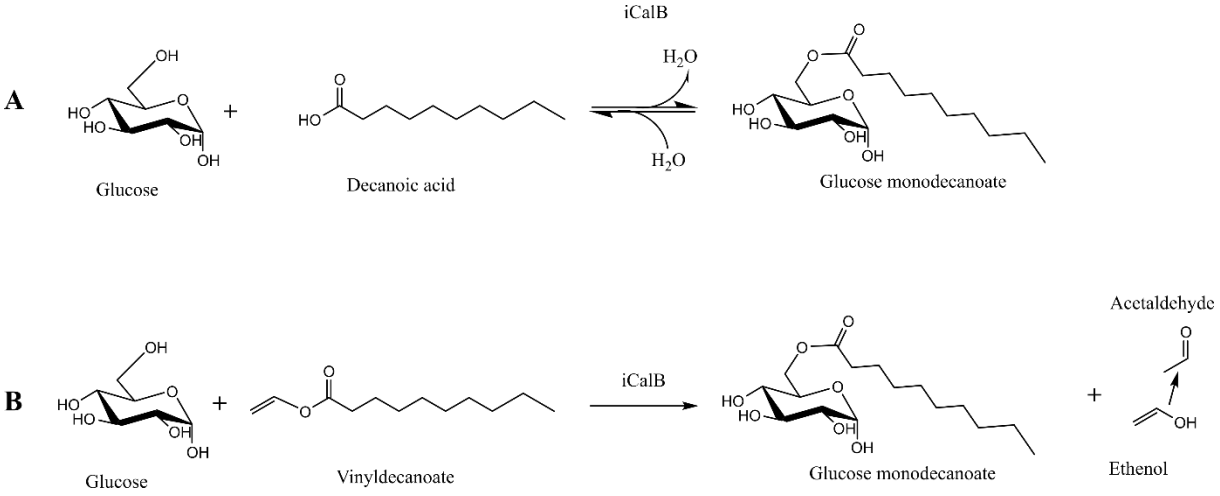

Figure 9. Reaction scheme of glucose monodecanoate synthesis. A: Esterification reaction; B: transesterification reaction. iCalB: immobilized *Candida antarctica* lipase B. DES: Deep eutectic solvents.

3.2. Materials and Methods

3.2.1. Materials

Glucose, (-)-menthol and all solvents (HPLC grade) were acquired from Carl-Roth (Karlsruhe, Germany). Lipase B from *Candida antarctica*, immobilized on acrylic resin (iCalB), was purchased from Strem Chemicals (Strem chemicals Europe, Germany). Vinyl decanoate and decanoic acid were acquired from Tokyo Chemical Industry Co., Ltd. (TCI Europe, Belgium). 6-Decanoyl-D-glucose was purchased from Sohena (Tübingen, Germany).

3.2.2. Viscosity Measurements

Viscosity measurements were performed using a Physica MCR 101 viscosimeter (Anton Paar, Ostfildern, Germany) with double gap geometry (DG26.7) at temperatures of 20 °C and 50 °C. Measurements were conducted at shear rates of 2–100 s^{-1}.

3.2.3. Water Content Analysis

The water content was determined by Karl-Frischer titration using a TitroLine 7500 KF trace from SI Analytics (Mainz, Germany) at 20 °C using Aquastar CombiCoulomat fritless (Merck Millipore, Darmstadt, Germany) as an analyte. A water standard of Merck Millipore (Darmstadt, Germany) was used to test the titrator before the measurements.

3.2.4. Glycolipid Synthesis

Decanoic acid and (-)-menthol were mixed in a molar ratio of 1:1 in a glass bottle at 80 °C for 1 h until a homogenous liquid was obtained. For all reactions, 1 mL DES was transferred to 5 mL reaction tubes, and glucose (final concentration 0.5 M) was added. For the transesterification reactions, 0.5 M vinyl decanoate was supplemented additionally. For the reaction with additional decanoic acid, decanoic acid (final concentration 0.5 M) was supplemented after the DES production. Finally, 20 mg/mL iCalB were added to start the reaction. The tubes were mixed in a rotator with a vortex mixer (program U2) from neoLab (Heidelberg, Germany) at 60 rpm and 50 °C. Samples for HPLC measurements were taken at distinct timepoints, diluted with ethyl acetate and analyzed by HPLC-ELSD.

3.2.5. Initial Reaction Velocity

To determine the initial reaction rates, glycolipid synthesis was carried out as described above (50 °C, 90 rpm), and samples for HPLC analysis were taken after 4 h.

3.2.6. Influence of Enzyme Concentration

In order to evaluate the impact of enzyme amount on product formation and initial reaction rates, the following enzyme concentrations were investigated without changing any other parameters: 5 mg/mL, 10 mg/mL, 20 mg/mL, 40 mg/mL and 60 mg/mL iCalB.

3.2.7. Optimization of Glucose Amount

To examine the effect of the added glucose amount on initial reaction rates, an enzyme concentration of 20 mg/mL was applied while keeping the other reaction parameters unchanged. The following final glucose concentrations were tested: 0.25 M, 0.5 M, 0.75 M, 1.0 M, 1.25 M and 1.5 M. After 4 h, samples were taken to determine the initial reaction velocity.

3.2.8. Reusability of Enzyme

Reusability of iCalB was tested for esterification as well as transesterification reactions. For esterification, 0.5 M glucose, 20 mg/mL iCalB and 1 mL DES were applied; for transesterification, 1.5 M glucose, 0.5 M vinyl decanoate and 20 mg/mL iCalB were added to 1 mL DES. After 24 h synthesis, the mixture was filtered using a Büchner funnel. The enzyme was washed three times with ethyl acetate to get rid of remaining DES and three times afterward with distilled water to get rid of remaining sugar. Then the enzyme was freeze-dried with a DW-10N freeze drier from Drawell (Shanghai, China) for 48 h. Afterward, the dried enzyme was reused for another synthesis. The conversion in the first cycle was set to 100 % to calculate the residual activity of the enzyme in the following cycles.

3.2.9. HPLC-ELSD-Analysis

Samples were analyzed by reversed-phase HPLC according to the method described by Hollenbach et al., 2020 (103). The HPLC system was equipped with a Kinetex EVO C18 column (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 using an Agilent (Waldbronn, Germany)1260 series liquid chromatograph equipped with a quaternary pump, an autosampler and a column oven. Analytes were detected using an evaporative light scattering detector from BÜCHI Labortechnik (Essen, Germany). The retention times were 2.1 min for glucose and 2.7 min for glucose monodecanoate.

3.2.10. Statistical Analysis

Results are given as mean ± standard deviation ($n = 3$). Statistical data analysis was performed by two-way ANOVA and Tukey test. For this, the software OriginPro 9.6 (version 2019; OriginLab, Nothampton, Massachusetts, USA) was used. Results were considered as significant if p-value was <0.05.

3.3. Results

The main purpose of this study was to examine the suitability of a hydrophobic (-)-menthol: decanoic acid DES as a medium for glycolipid synthesis and to compare the results with the synthesis in hydrophilic DES. Additionally, the influence of different reaction parameters was investigated to characterize the esterification as well as the transesterification reaction in this DES.

3.3.1. Reaction Time Course

The reaction time course was monitored with emphasis on the water released during esterification reaction, as water content is a crucial parameter for reversed hydrolysis. Within the transesterification reaction, water is not released. Although it might be interesting to determine water content for both reactions, water content measurements were only feasible for the reactions which did not contain vinyl decanoate as acetaldehyde interferes with the analysis. Therefore, water content can only be presented for the esterification reaction.

Glucose monodecanoate concentration and water content were both rising in the first 24 h of reaction (**Figure 10A**). Subsequently, glucose monodecanoate production stagnated while water content increased further.

In order to investigate whether the stagnation of product formation could be caused by a limited availability of the fatty acid in the DES, a further reaction set-up was carried out in which decanoic acid was supplemented in addition to the decanoic acid contained in the DES. The reaction progression remained unaltered when 0.5 M decanoic acid was added, causing no difference in comparison to the reaction without fatty acid addition (**Figure 10B**).

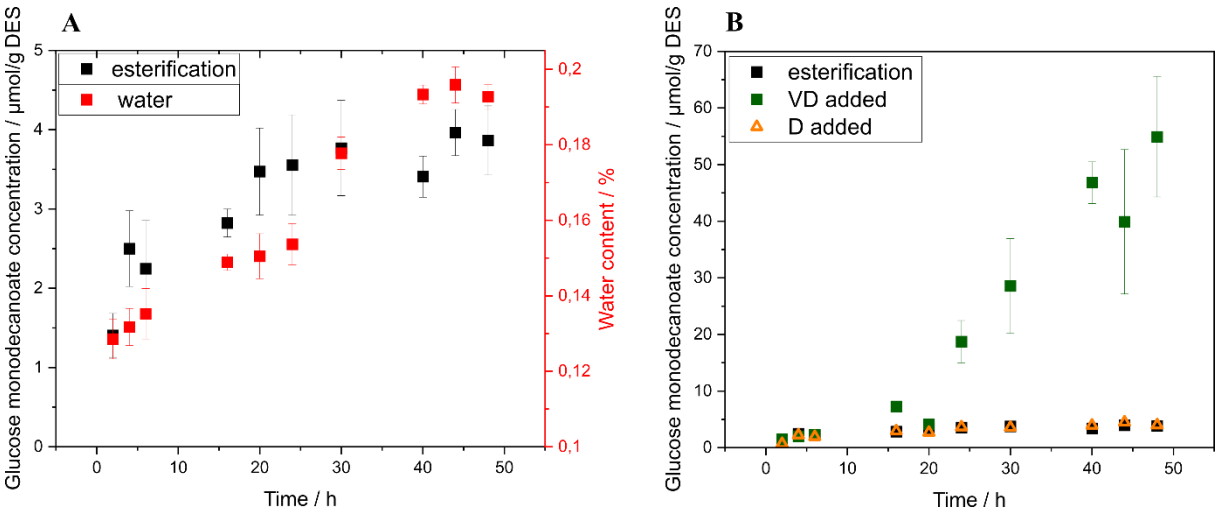

Figure 10. Reaction time course of glucose monodecanoate synthesis. A: Glucose monodecanoate synthesis and water release during esterification reaction; B: comparison of glucose monodecanoate synthesis in esterification, in esterification reaction with added decanoic acid (D added) and in transesterification reaction (VD added). D and VD were added after deep eutectic solvents (DES) production and before enzyme addition. D: decanoic acid; VD: vinyl decanoate.

Transesterification reactions in which vinyl decanoate was applied additionally showed a lag phase over the first 16 h of reaction time. Due to this lag phase, the transesterification reaction and esterification reactions in which decanoic acid was used simultaneously for DES formation and as substrate did not differ in product formation rate within the first 6 h of reaction (**Figure 10B**). Subsequently, however, the product formation rates are significantly higher in transesterification reactions. Product yield after 24 h was 18.73 ± 3.73 µmol/g DES in contrast to 3.55 ± 0.63 µmol/g DES for esterification. At 48 h, the difference between both reaction set-ups was even higher, with 54.93 ± 10.66 µmol/g DES with added vinyl decanoate compared to 3.86 ± 0.43 µmol/g DES without. No product formation nor side product formation was observed in the negative controls (without addition of glucose). Chromatograms clearly indicate that (-)-menthol decanoate was not formed under the conditions applied within 120 h using iCalB as enzyme (**Appendix: Figure A3**).

3.3.2. External Mass Transfer

The viscosity of the (-)-menthol: decanoic acid DES is 20.5 ± 0.05 mPa·s at 20 °C and 5.1 ± 0.09 mPa·s at 50 °C. The viscosity of water is 1.0 ± 0.01 mPa·s at 20 °C and 0.5 ± 0.01 mPa·s at 50 °C. Thus, mass transfer limitation is possible due to the higher viscosity of the DES compared to water. Hence, mixing is a crucial parameter. Therefore, the initial reaction velocity was investigated as a function of the agitation rate to evaluate external mass transfer. An increase in agitation rate increased the initial reaction velocity neither in the reaction with only free fatty acid nor in the reaction with added vinyl decanoate (**Figure 11**).

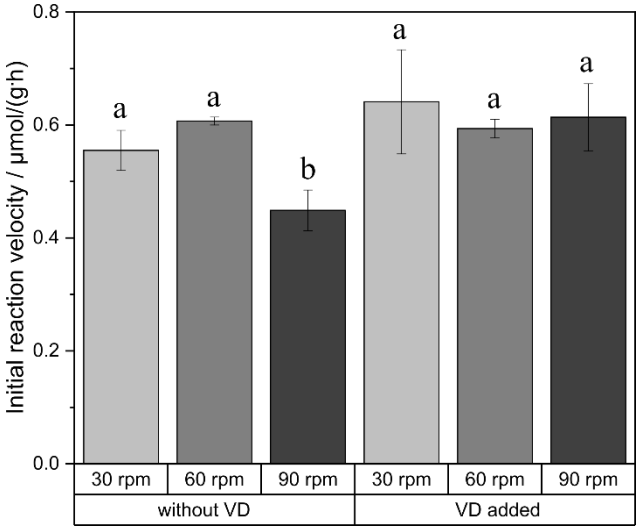

Figure 11. Initial reaction velocity in relation to the agitation rate. a, b indicate statistically significant differences. VD: vinyl decanoate.

3.3.3. Effect of Enzyme Concentration

In order to find the best reaction conditions, enzyme concentration was evaluated. For the reaction without vinyl decanoate, a maximum was observed for the enzyme concentration (**Figure 12**). Initial reaction velocity increased with rising enzyme concentration up to 20 mg/mL. However, at higher enzyme concentrations, the initial reaction rate dropped. For the reactions with vinyl decanoate, this trend was not observed. Instead, enzyme concentrations from 10 mg/mL to 60 mg/mL resulted in the same initial reaction velocity as the standard reaction without vinyl decanoate at 20 mg/mL enzyme concentration.

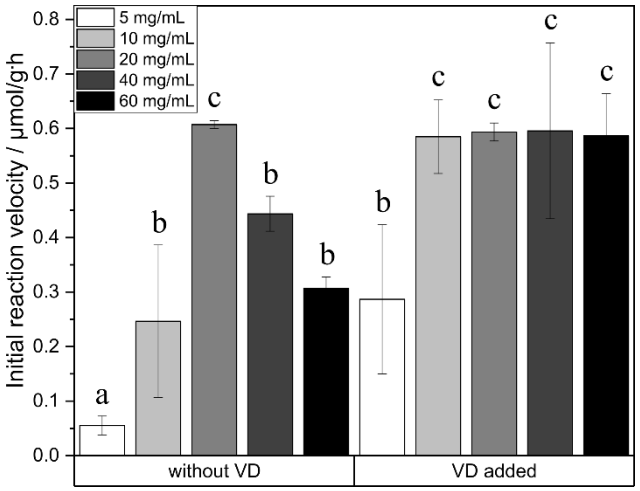

Figure 12. Impact of enzyme concentration on initial reaction velocity. a, b, c show statistically significant differences. VD: vinyl decanoate.

3.3.4. Effect of Initial Glucose Amount

To evaluate suitable reaction parameters, the addition of glucose was also investigated. A glucose amount of 0.25 M instead of 0.5 M resulted in lower initial reaction velocity for both reactions, the one without vinyl decanoate as well as for the one with added vinyl decanoate (**Figure 13**), respectively. Further increase in glucose amount had no influence on the initial reaction velocity of the esterification reaction without vinyl decanoate. In contrast, the reaction

rate of the synthesis with added vinyl decanoate was significantly enhanced by increasing sugar amounts. Increasing the glucose amount to 1.5 M improved the glucose monodecanoate yield significantly to 164.27 ± 9.98 µmol/g DES at a reaction time of 24 h.

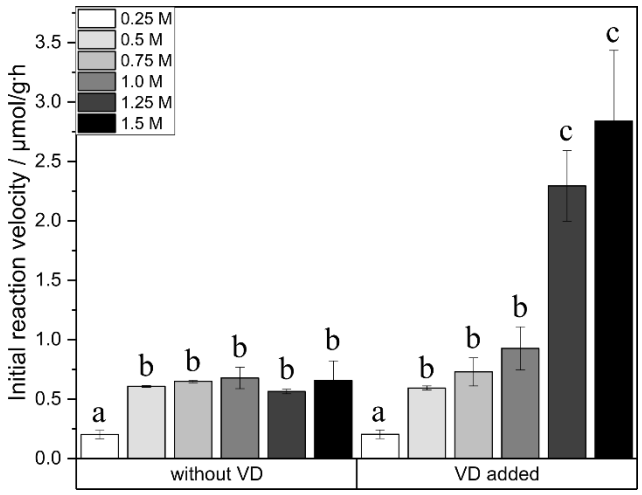

Figure 13. Initial reaction velocity in relation to different glucose amounts. a, b, c show statistically significant differences. VD: vinyl decanoate

3.3.5. Reusability of Enzyme

The reusability of the enzyme is of interest especially for industrial applications as the catalyst costs are getting negligible with a rising number of reaction cycles. Therefore, we investigated the development of enzyme activity over several reaction cycles. In preliminary experiments, the lyophilization time of the washed enzyme was evaluated by measuring the water content. The water content of the fresh enzyme formulation was 1.55 ± 0.06 %. After 24 h of lyophilization of the washed enzyme, the water content was 1.82 ± 0.10 %. However, an extended lyophilization time (up to 120 h) did not reduce the water content further.

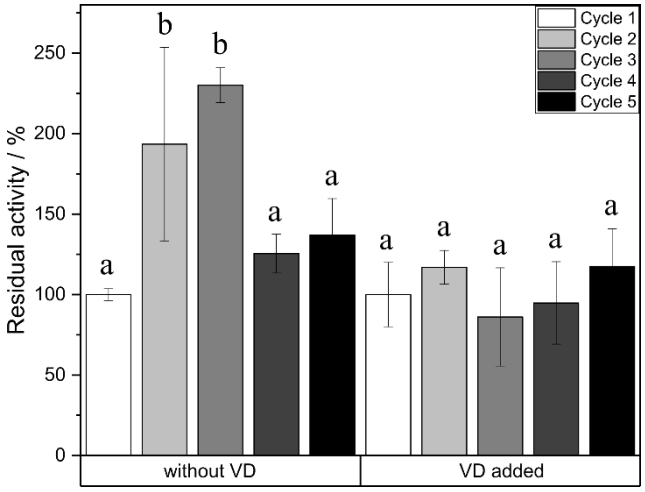

Figure 14. Residual activity of the enzyme in relation to the reaction cycle. Yield of reaction cycle 1 after 24 h was set to 100 %. a, b show statistically significant differences. VD: vinyl decanoate.

For the esterification reaction, no loss of activity was observed over five reaction cycles (**Figure 14**). Cycles 2 and 3 showed a significantly higher yield after 24 h of reaction compared

to cycle 1. The relative activity of the enzyme remained the same over five cycles for the reaction with added vinyl decanoate.

3.4. Discussion

In this study, it was shown for the first time that a hydrophobic DES containing (-)-menthol and decanoic acid is suitable for enzymatic glycolipid synthesis. Remarkably, glucose monodecanoate yields in the investigated hydrophobic (-)-menthol: decanoic acid DES are 20 to 1000 times higher than those reported by Hollenbach *et al.*, 2020, in hydrophilic DES (**Table 6**) (103). The increase in productivity is likely caused by the difference in solvent polarity as solvent polarity is already reported to have an effect on glycolipid synthesis in organic solvents (31,32). In IL, it is also reported that medium polarity is most appropriate to dissolve sugar as well as fatty acids (98). The solvatochromic π^* is a measure of polarizability and dipolarity of solvents. The π^* value is much lower for the (-)-menthol: decanoic acid DES (0.35) than for hydrophilic DES containing choline chloride and urea (1.192) or choline chloride and glucose (1.161) (44,45). Therefore, polarity can be assumed as a major parameter in glycolipid synthesis in DESs.

Table 6. Comparison of glucose monodecanoate yields in (-)-menthol: decanoic acid DES and in hydrophilic DES.

Different DES	Glucose Monodecanoate Yield (24 h)
Choline chloride: urea DES with VD	0.15 μmol/g DES (0.03 %) (103)
(-)-menthol: decanoic acid DES	3.55 μmol/g DES (0.71 %)
(-)-menthol: decanoic acid DES with VD (0.5 M glucose)	18.73 μmol/g DES (3.75 %)
(-)-menthol: decanoic acid DES with VD (1.5 M glucose)	164.27 μmol/g DES (10.95 %)

VD: vinyl decanoate; yields [%] were calculated based on the glucose concentration, with a theoretical yield of c (glucose) = c (glucose monodecanoate) = 100 %.

The hydrophobic and hydrophilic DES differ not only in their polarity but also in their viscosity. The viscosity of the hydrophobic DES is 10–30 times lower than those reported for hydrophilic DES (103). However, external mass transfer limitation can be excluded by sufficient mixing. Thus, viscosity seems to be only of minor importance in glycolipid synthesis in DES. For downstream processing, however, the lower viscosity of the (-)-menthol: decanoic acid DES compared to the hydrophilic DES might be beneficial. The different strengths and natures of the hydrogen bonding network in the different DESs contribute to their different viscosities, as well as likely to their different performances. The hydrogen bond acidity α and the hydrogen bond basicity β are both lower for the presented hydrophobic DES than for the hydrophilic ones (44,45).

Negative controls without addition of glucose were performed to exclude the formation of (-)-menthol decanoate as this reaction was reported in (-)-menthol: fatty acid DES with *Candida rugosa* lipase (109). No product formation was detected in these negative controls. iCalB prefers primary hydroxy groups (90,113,150). Menthol has a secondary hydroxy group with additional steric hindrance due to an adjacent isopropyl group.

Water content is reported to be a crucial parameter in reversed hydrolysis (35,44,76). Water is released during esterification reaction (**Figure 9A**). Since water is consumed again in hydrolysis, the reaction equilibrium is shifted to the reactants' side once a certain water content

is reached (35,76,151,152). During the esterification reaction, water content raised from 0.13 % to 0.19 % until stagnation as water is formed within the reaction.

Another possible limitation of the reaction might be bare availability of decanoic acid for the reaction since decanoic acid acts simultaneously as hydrogen bond donor in the DES. Strong associations between substrates and the hydrogen bond network of the DES are reported that cause a low availability of substrates (153). However, limited availability of decanoic acid in the DES for the enzymatic reaction was excluded by addition of free fatty acid to the reaction, which caused no difference in the pattern of the reaction. Therefore, the increasing water content is most likely the limiting factor of the reaction with exclusively free fatty acid, leading to an equilibrium between synthesis and hydrolysis of glucose monodecanoate.

The transesterification reaction using vinyl decanoate as additional substrate resulted in 6 times higher yields than the esterification even though there is no difference in the reaction time course during the first 6 h. During transesterification reaction, water is not formed, which might shift the equilibrium to hydrolysis. Instead, ethenol is formed that tautomerizes to its corresponding aldehyde, acetaldehyde, and evaporates (**Figure 9B**). Thus, the reaction is shifted towards the product side. This is likely the reason why the yield of the transesterification reaction is higher than that of the esterification reaction.

External mass transfer limitation can be excluded under the conditions used as the initial reaction velocity remains unaltered at increased reaction rates. The maximum in the initial reaction velocity depending on the agitation rate might be due to a loss of enzyme activity at higher shaking rates. An optimum in agitation rate was also observed by Zhao *et al.*, 2011, in mono- and diglyceride synthesis (154). The absence of that maximum for the synthesis with added vinyl decanoate is most likely because half the amount of enzyme leads to the same initial reaction velocity as 20 mg enzyme/mL for the transesterification reaction. The investigations of the enzyme concentration showed an increased initial reaction velocity with increasing enzyme concentration up to 10 mg/mL. At higher concentrations, a stagnation of the initial reaction velocity was observed due to saturation. However, an optimum in enzyme concentration was determined for the esterification reaction. Due to the water formation within this reaction, an increased enzyme concentration might lead to a higher velocity of the hydrolysis reaction, which causes lower product yields. Nevertheless, the higher enzyme concentration also led to an increased initial water concentration due to the water within the matrix of the enzyme formulation. The initial water content at an enzyme concentration of 60 mg/mL is in the range of the water content at a steady state of the esterification reaction when using an enzyme concentration of 20 mg/mL. Therefore, the inhibitory effect could also be ascribed to the water content. Similar findings were already observed in glucose ester synthesis in organic solvents (155).

The initial reaction velocity increased with increasing glucose amounts, although glucose had not been completely dissolved in any of the applied concentrations. The observed results might be due to a faster glucose dissolution at higher glucose amounts. This effect could only be observed for the transesterification reaction; for the esterification reaction, though, a stagnation occurred at 0.5 M glucose. Transesterification might be faster than esterification because of its thermodynamic advantage. Therefore, the amount of glucose has a stronger influence on transesterification than on esterification.

The investigations on the reusability of the enzyme over five reaction cycles showed no significant loss of activity for the esterification reaction nor for the transesterification reaction. Pre-incubation of iCalB in the hydrophilic DES, choline chloride: glycerol and choline chloride: urea was reported to cause a significant loss of activity of 70 % and 38 % (153). The higher stability of iCalB in the (-)-menthol: decanoic acid DES might be due to the lower polarity of the latter. Solvent polarity is reported to have a relation to enzyme activity and stability as more polar solvents strip off hydration water from the enzyme (78,79).

3.5. Conclusions

The aim of this study was to investigate the applicability of a hydrophobic (-)-menthol: decanoic acid DES for glycolipid synthesis. The glucose monodecanoate yields were significantly improved by using the newly introduced DES compared to the hydrophilic DES used so far. The polarity of the used solvent was identified as crucial for glycolipid productivity. Furthermore, the reaction was also possible with free fatty acids instead of the thermodynamically preferred reaction with vinylated fatty acids. Therefore, the additional reaction step generating vinylated fatty acids can be omitted, as well as the highly volatile side product acetaldehyde. Moreover, the enzyme showed high stability and reusability in (-)-menthol: decanoic acid DES without loss of activity for at least five reaction cycles.

4. Structure-Function Relationship

This chapter is partly based on the publication:

Interfacial and Foaming Properties of Tailor-Made Glycolipids – Influence of the Hydrophilic Head Group and Functional Groups in the Hydrophobic Tail
Rebecca Hollenbach, Annika Völp, Ludwig Höfert, Jens Rudat, Katrin Ochsenreither, Norbert Willenbacher, Christoph Syldatk
Molecules, 2020, 25 (17), 3797
doi: 10.3390/molecules25173797
Published: 20.08.2020

Authors Contribution:

Rebecca Hollenbach: Design of the study, investigation, formal analysis, supervision of Ludwig Höfert, writing original draft, review and editing of the final manuscript

Annika Völp: investigation, review and editing of the final manuscript

Ludwig Höfert: investigation

Jens Rudat: supervision (initiated the cooperation), conceptual advice, review and editing of the final manuscript

Katrin Ochsenreither: conceptual advice, review and editing of the final manuscript

Norbert Willenbacher: conceptual advice, supervision, review and editing of the final manuscript

Christoph Syldatk: conceptual advice, supervision, review and editing of the final manuscript

4.1. Introduction

Foams are thermodynamically unstable systems of bubbles dispersed in a solution stabilized by surfactants which can have a wide range of possible applications, e.g., in agriculture, cosmetics, food, fire-fighting, oil recovery and wastewater treatment (156,157). Foams become destabilized by drainage, coarsening and coalescence (156,158,159). These destabilizing mechanisms are related to dynamic interfacial tension, interfacial elasticity and interfacial viscosity (160–163). Therefore, foam characteristics, such as foam stability, coarsening rates and bubble size distribution, strongly depend on the surfactant used for stabilization. Surfactants are amphiphilic molecules of a hydrophilic and a hydrophobic moiety. Therefore, they adsorb at interfaces and stabilize them by lowering interfacial tension as well as providing a barrier against aggregation and coalescence. Certain surfactants may also induce interfacial viscoelasticity.

Glycolipids are a class of surfactants consisting of a lipid moiety linked to a carbohydrate. They are produced chemically or biotechnologically, either by fermentation or by enzymatic synthesis. Biotechnological production has the advantage over chemical production of milder reaction conditions, as neither high temperatures nor toxic catalysts nor protection and deprotection steps are required (30). The structural diversity of the fermentatively produced glycolipids is limited by the metabolism of the microorganisms, whereas in enzymatic synthesis there are theoretically no limits to this diversity (30). Hence, enzymatic synthesis is a promising strategy for production of tailor-made glycolipids. Knowledge of the structure–function relationship is essential to select suitable head and tail groups for the respective application. While numerous studies dealt with the foaming properties of petrochemically derived surfactants, only few studies are available on glycolipid applications in foams, although glycolipids are biodegradable and less harmful to the environment than petrochemical surfactants, qualifying them as sustainable alternatives (5,7–11). Another advantage of glycolipids over conventional surfactants is the temperature-insensitivity of their physicochemical properties which allows for applications over a broad temperature range (164,165). Glycolipids are non-toxic and skin friendly, and some of them exhibit antimicrobial activity (5,11,166,167). Hence, glycolipids are highly interesting for applications in food, cosmetics and pharmaceutics (3,19,20).

For alkyl glycosides, it is known that foam stability of molecules with a monosaccharide as head group is best with a C10 tail group, while shorter and longer chain length decreases foam stability (168,169). However, alkyl glycosides have a higher skin irritation potential than glycolipids with an ester bond instead of the ether bond (167). Thus, sugar acylates have a broader range of applications. Zhang *et al.* reported that acylated monosaccharides with a C10 fatty acid chain exhibit higher foaminess, higher foam stability and lower critical micelle concentration (CMC) than laurates (14). Therefore, glycolipids with an ester bond and tail groups of 10 carbons were enzymatically synthesized in this study.

Head groups are reported to have only minor effects on interfacial tension and CMC (169,170), but alkyl glucosides with different head groups showed differences in foam stability (169). However, the knowledge of the influence of head groups on foaming properties is limited to foam stability tests in a time range of 5 min.

Foaming properties are related to interfacial properties like dynamic interfacial tension and interfacial rheology (156,158–163,171). Foam rheology has been shown not only to depend on Laplace pressure within the bubbles and gas volume fraction but also on interfacial elasticity (172). Interfacial elasticity and interfacial viscosity also have an impact on foam stability. A higher interfacial viscosity results in foams with higher resistance against coarsening and foam rupture leading to slower foam decay (159,162,163). However, only the study of Razafindralambro *et al.* addresses interfacial rheology of two glycolipids with different head groups, i.e., glucose octanoate and octyl glucuronate (171).

To the best of our knowledge, a comparative study on interfacial and foaming properties of different glycolipids focusing on acylated monosaccharides with a specific tail length has not been described in the literature yet. In this study, seven glycolipids with C6 head groups and C10 tail groups were enzymatically synthesized and evaluated (**Figure 15**). Four different sugar(-derivatives) were applied as a head group, namely the aldose glucose, the ketose sorbose, the uronic acid glucuronic acid and the alditol sorbitol. Fatty acid tails were either saturated, unsaturated or branched. In this study, foaming properties, including foam decay, transient gas volume fraction and mean bubble diameter as well as foam rheology, were related to interfacial dilatational viscoelasticity and glycolipid structure.

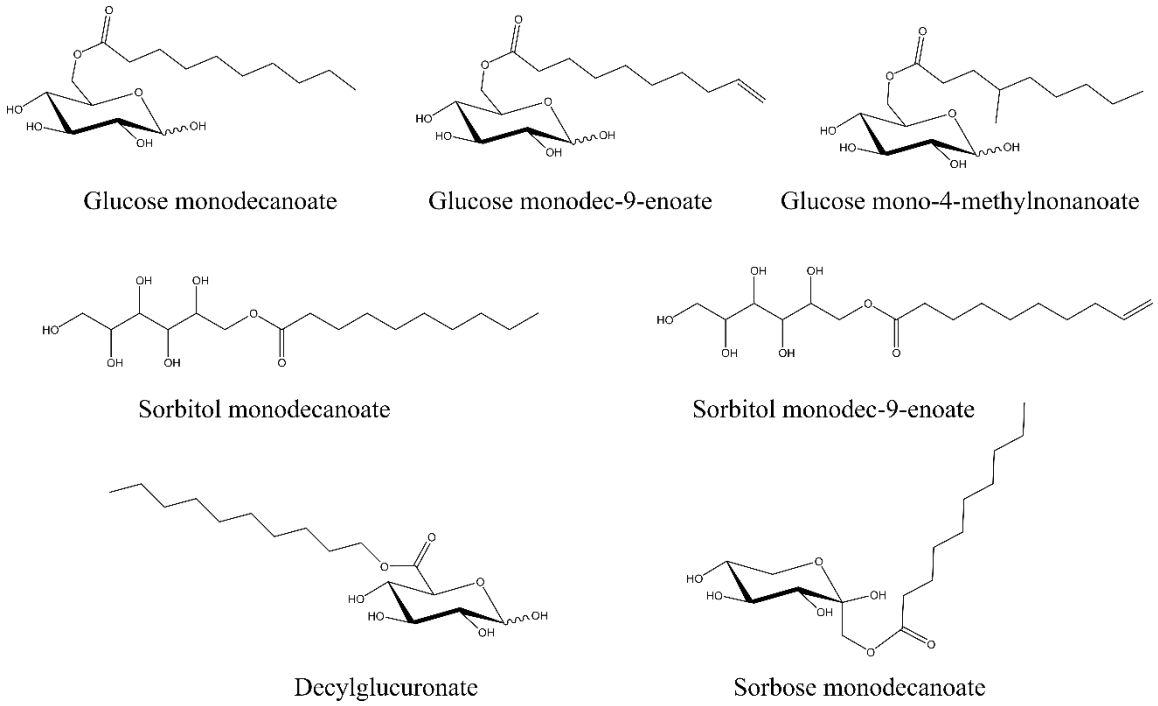

Figure 15. Structures of the investigated glycolipids.

4.2. Materials and Methods

4.2.1. Materials

Lipase B from *Candida antarctica*, immobilized on acrylic resin (iCalB) was purchased from Strem Chemicals (Strem chemicals Europe, Kehl, Germany). Vinyl decanoic acid and dec-9-enoic acid were acquired from Tokyo Chemical Industry Co., Ltd. (TCIEurope, Eschborn, Germany). 4-methyl nonanoic acid and glucuronic acid were purchased from VWR (Radnor, PA, USA). Glucose, sorbitol and all solvents (in HPLC grade) were acquired from Carl-Roth

(Karlsruhe, Germany). Sorbose was a kind gift from Givaudan (Paris, France). 6-Decanoyl-D-glucose was purchased from Sohena (Tübingen, Germany).

4.2.2. Synthesis of Glycolipids

Substrates were mixed in equimolar ratio, 0.5 M sugar(derivate) and 0.5 M (vinyl-)fatty acid, and 10 mg/mL iCalB in a 250 mL round bottom flask in 100 mL acetone. iCalB prefers primary hydroxyl groups and therefore esterification takes place at the primary hydroxyl group of the sugar(derivative) (90,113,173). The samples were shaken at 50 °C and 600 rpm in a Laborota 4000 rotatory evaporator (Heidolph, Schwabach, Germany) at atmospheric pressure for 48 h. For the synthesis of glucose monodecanoate glucose and vinyldecanoate were used as substrates, for glucose monodec-9-enoate glucose and 9-decenoic acid, for glucose mono-4-methyl-nonanoate glucose and 4-methyl-nonanoate, for sorbitol monodecanoate sorbitol and vinyldecanoate, for sorbitol monodec-9-enoate sorbitol and 9-decenoic acid, for decylglucuronate glucuronic acid and decanol and for sorbose monodecanoate sorbose and vinyldecanoate.

4.2.3. Purification of Glycolipids

The obtained glycolipids were filtrated with a Büchner funnel and the filtrate was washed three times with ethyl acetate. The glycolipid containing solvent was evaporated with a rotatory evaporator at 40 °C and 240 mbar. Solids were subsequently purified by flash chromatography using a Reveleris Prep system from Büchi Labortechnik GmBH (Essen, Germany) and a Flash Pure Silica column (40 g, 53–80 Å). Mobile phase was made of chloroform (A) and methanol (B). A gradient was used for separation of products and residual substrates: starting from 100 % A, a linear gradient was applied to 96 % A and 4 % B within 2 min. This ratio was held for 9 min, followed by another linear gradient to 90 % A and 10 % B in 2 min. This ratio was held for 6 min. Afterwards a linear gradient to 75 % A and 25 % B in 2 min was applied, and this ratio was held for 4 min, followed by a linear gradient to 100 % B in 2 min, and this was held for 6 min. Peaks were collected and fractions controlled by TLC. Therefore, 5 µL of samples were spotted on Alugram Xtra SIL G plate from Machery-Nagel (Düren, Germany). For elution a mobile phase of chloroform: methanol: acetic acid was used (65:15:2, by vol). Compounds were visualized by anis aldehyde dying (anis aldehyde: sulfuric acid: acetic acid 0.5:1:100, by vol). Product containing fractions were collected and solvents were again evaporated with a S-Concentrator BaVC-300H from Helmut Saur Laborbedarf (Reutlingen, Germany). The purity of the products was checked by HPLC-ELSD.

4.2.4. HPLC-ELSD

HPLC analysis was performed according to Hollenbach *et al.* (2020) using a Kinetex EVO C18 (2.6 µm, 250 × 4.6 mm) from Phenomenex (Aschaffenburg, Germany) with an accompanying guard column (4 x 3.0 mm ID) of the same phase using an Agilent (Waldbronn, Germany) 1260 series liquid chromatograph equipped with a quaternary pump, an autosampler and a column oven (103). For detection, an evaporative light scattering detector from BÜCHI Labortechnik (Essen, Germany) was used. Mobile phase was a gradient of acetonitrile (A) and water (B) with a total flow rate of 1 mL/min. This method reliably separates monoesters from substrates and by-products such as diesters (103). Only products with a purity of at least 95 % determined by the area % of the HPLC chromatograms were used for further investigations.

4.2.5. Determination of Interfacial Tension

Interfacial tension was determined with a Lauda Tensiometer TD1 (Lauda-Königshofen, Germany) by the Du Noüy-ring method. Before the measurement, the tensiometer needed to be prepared by calibrating with a 500 mg calibration weight. A test vessel was filled with at least 2 mL glycolipid solution and placed on the stage of the tensiometer. A Du Noüy-ring (19.1 mm diameter) was submerged at least 2-3 mm below the solution surface. After an equilibration time of 15 min, the measurement was started by lowering the stage manually. The maximum normal force before the lamella formed between ring and solution breaks is the uncorrected interfacial tension σ_{unc}. The absolute interfacial tension σ_{abs} is obtained by multiplying σ_{unc} with a correction factor *f*. The correction factor f for the used ring was calculated as follows (174,175):

$$f = 0.8759 + \frac{0.0009188}{\rho}, \quad (1)$$

where ρ is the density of the test liquid.

The surface excess concentration Γ and the molecular area A were calculated according to Blecker *et al.* (2002) using Gibbs adsorption isotherm (176):

$$\Gamma = \frac{1}{RT} \times \left(\frac{d\sigma}{d\ln C}\right), \quad (2)$$

$$A = \frac{1}{\Gamma \times N}, \quad (3)$$

where R is the universal gas constant, T is the temperature in K, σ is the interfacial tension, C is the surfactant concentration, and N is the Avogadro number.

4.2.6. Dynamic Interfacial Tension and Interfacial Rheology Measurements

The dynamic interfacial tension and interfacial rheology of all solutions were determined using a pendant drop tensiometer (PAT1, Sintaface, Berlin, Germany). A drop of the respective solution with a surface area of 20 mm^2 was produced from a cannula with 1 mm inner diameter. The interfacial tension was calculated from the drop shape over a period of 10,000 s maintaining a constant surface area. The surface area was then oscillatorically dilated for at least 10 oscillations with an amplitude of 2 mm^2, followed by a 15-min oscillation pause at a constant surface area. The oscillation frequencies were 0.05, 0.1, 0.33, 0.5 and 0.67 Hz. The drop surface was dilated three times with oscillations of each frequency. The interfacial tension, interfacial viscosity, and interfacial elasticity were determined as described in Loglio *et al.* (177). The respective mean value and deviation was calculated from two measurements with independently prepared solutions. The equilibrium interfacial tension and the time t_{eq}, when equilibrium was reached, were taken when the interfacial tension changes became smaller than the deviation.

4.2.7. Foam Generation

A 50 mL VitaPor suction filter funnel (Por.4, 10–16 µm) from ROBU Glasgeräte GmbH (Hattert, Germany) was used for foam generation. 16 mL of surfactant solution

(concentration = 2 × CMC) were filled into the suction filter funnel. Foam formation was initiated by introducing nitrogen with a gas flow of 60 mL/min through the funnel outlet. As soon as a foam height of 5.3 cm was reached, the nitrogen flow was stopped and the measurements were started.

4.2.8. Foam Height Measurements

Foam height was measured over a period of 60 min using a scale at the outside of the filter.

4.2.9. Bubble Size Distribution Measurements

Bubble sizes were analyzed using a VHX-950F microscope equipped with a VH-B55 endoscope both supplied by Keyence Deutschland GmbH (Neu-Isenburg, Germany). The endoscope, covered with a 90° angle mirror tube and inserted into a customized optical glass cuvette, was placed at a height of 22 mm above the filter. Pictures were taken every 15 s over a period of 10 min. For illumination the spotlight of a KL 1500 LCD goose neck lamp from Schott AG (Mainz, Germany) was placed at the outer wall of the filter funnel. The endoscopic pictures were evaluated using a software tool written in Matlab® (MathWorks®, Natick, MA, USA) based on a template matching method as described by Völp *et al.* (178). Bubble size distribution was analyzed in triplicates in freshly produced foams.

Coarsening rate Ω was obtained from the slope of square Sauter diameter versus time plots ($\Omega = \frac{\mathrm{d}D^2}{\mathrm{d}t}$) according to Briceño-Ahumada *et al.* (159). Coarsening rates were calculated in a time range from 100–600 s, and correlation coefficients were at least 0.96.

4.2.10. Determination of Gas Volume Fractions

Conductivity was measured using a SevenCompact conductivity meter equipped with an Inlab® 738 ISM four-electrode conductivity sensor from Mettler-Toledo (Schwerzenbach, Switzerland). The sensor was placed 22 mm above the filter membrane. The conductivity of the glycolipid solution was measured before foaming. The foam conductivity was measured every 15 s over a period of 10 min. The relative conductivity κ_{rel} was calculated by (4):

$$\kappa_{\mathrm{rel}} = \frac{\kappa_{\mathrm{foam}}}{\kappa_{\mathrm{solution}}}, \tag{4}$$

where κ_{foam} is the conductivity of the foam and $\kappa_{solution}$ is the conductivity of the glycolipid solution.

The gas volume fraction φ was calculated as described by Feitosa *et al.* (179):

$$\varphi = 1 - \frac{3 \times \kappa_{\mathrm{rel}} \times (1+11 \times \kappa_{\mathrm{rel}})}{1 + 25 \times \kappa_{\mathrm{rel}} + 10 \times \kappa_{\mathrm{rel}}^2}, \tag{5}$$

4.2.11. Determination of Shear Moduli

The shear modulus of the foams was determined using a RheoScope 1 rotational rheometer from Thermo Fischer Scientific (Karlsruhe, Germany) equipped with a plate-plate geometry with a diameter of 60 mm, covered with sandpaper (grit 40, average particle diameter 269 µm) to reduce wall slip effects. The gap height was set to 5 mm. A foam sample was prepared inside

the filter funnel and approximately 20 mL of foam were transferred onto the bottom plate of the rheometer using a spoon 20 s before it reached the desired foam age. The device set the gap automatically within 20 s and the measurement started. The foams were sheared in oscillation with a fixed frequency of 1 Hz and the stress amplitude increased stepwise from 0.01 to 20 Pa in 12 logarithmically distributed steps during a measuring time of 60 s. The shear modulus was obtained from the average real part G' of the shear modulus measured at stress amplitudes in the linear viscoelastic shear regime. In foams, the shear moduli are independent of the frequency typically in the range between 0.01 and 10 Hz (180) and since G' is determined in this regime it is termed shear modulus G_0.

4.2.12. Statistical Analysis

Results are given as mean ± standard deviation. Statistical data analysis was performed by two-way ANOVA and Tukey test using the OriginPro 9.6 (version 2019) software. Results were considered significant if p-value was <0.05.

4.3. Results

Tailor-made glycolipids synthesized and investigated in this study had a purity of at least 95 %. CMC was determined, and further measurements were conducted at glycolipid concentrations of twice the CMC. Dynamic interfacial tension, as well as interfacial elasticity and interfacial viscosity were determined as characteristic interfacial properties. Foam stability, i.e., transient foam height, bubble diameter and gas volume fraction, as well as foam shear modulus were analyzed for evaluation of the structure–function relationship of the different glycolipids.

4.3.1. Critical Micelle Concentration (CMC) and Dynamic Interfacial Tension

Critical micelle concentrations were determined for all glycolipids by Du Noüy ring method, and the results are summarized in **Table 7**. In general, glycolipids with a saturated fatty acid tail had significantly lower CMC values than those with monounsaturated fatty acid tails. The lowest CMC was determined for sorbitol monodecanoate (0.74 mM).

Table 7. Interfacial properties of the investigated glycolipids, including critical micelle concentration (CMC), equilibrium interfacial tension and molecular area.

Glycolipids	CMC in mM	Interfacial tension in mN/m [1]	t_{eq} in s	Molecular area in Å²/molecule
Glucose monodecanoate	1.5	25.5 ± 0.17 [a]	2400	26.3
Glucose monodec-9-enoate	3.0	28.5 ± 1.10 [b,c]	1100	30.7
Glucose mono-4-methylnonanoate	1.8	29.6 ± 1.90 [b]	300	39.4
Sorbitol monodecanoate	0.7	24.9 ± 0.84 [a]	2900	32.6
Sorbitol monodec-9-enoate	3.0	26.0 ± 0.13 [a,c]	700	42.1
Decylglucuronate	1.3	24.3 ± 0.63 [a]	100	33.7
Sorbose monodecanoate	1.0	25.0 ± 1.10 [a]	600	30.9

[1] (c = 2 × CMC). [a,b] indicate statistical significant differences; [c] indicates statistical significant difference to the corresponding glycolipid with saturated fatty acid tail. t_{eq} is the time when equilibrium interfacial tension is reached.

Dynamic interfacial tension measurements were performed using the pendant drop method in order to characterize the adsorption velocity of the glycolipids, which is known to have an impact on foaming properties. The equilibrium interfacial tension of the investigated glycolipids was in the range between 24 mN/m and 29 mN/m with the lowest interfacial tension of 24.3 ± 0.6 mN/m for decylglucuronate and the highest interfacial tension of 29.6 ± 1.9 mN/m for glucose mono-4-methylnonanoate (**Table 7**). Highest interfacial tension values were found for glycolipids with unsaturated or branched tail groups.

Decylglucuronate and glucose mono-4-methylnonanoate adsorbed much faster at the interface than the other investigated glycolipids, resulting in a faster reduction of the interfacial tension (**Figure 16**). Sorbitol monodecanoate had a significantly longer adsorption time than the other glycolipids (**Figure 16A**). Remarkably, the adsorption times of the glycolipids with a monounsaturated fatty acid tail were 2–4 times shorter than those of the corresponding saturated glycolipids (**Figure 16B**).

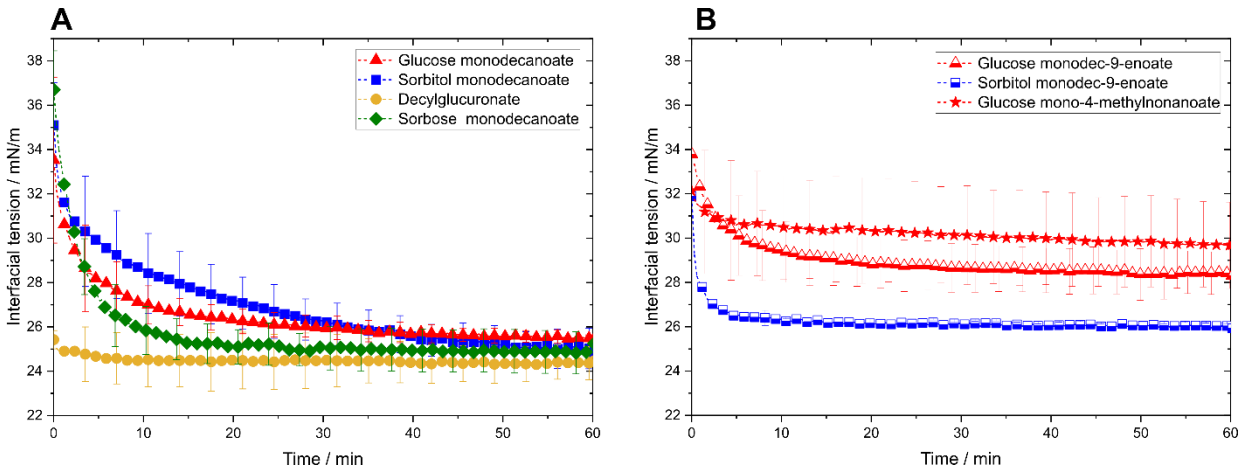

Figure 16. Dynamic interfacial tension of the investigated glycolipids vs. interface lifetime in pendant drop experiments. A: Comparison of the different head groups. B: Comparison of the different tail groups. Decylglucuronate reached equilibrium faster than glycolipids with glucose, sorbitol or sorbose head group. The branched glucose mono-4-methylnonanoate led to a faster reduction of interfacial tension than unbranched glycolipids.

4.3.2. Interfacial Rheology

Interfacial rheological properties, i.e., interfacial elasticity and interfacial viscosity are known to influence foam properties since they contribute to resistance against coarsening, coalescence and drainage (160–163).

Interfacial dilatational elasticity of the investigated glycolipids was significantly different depending on the structure of the glycolipid (**Figure 17A**). The interfacial elastic moduli of solutions of glycolipids with branched or monounsaturated fatty acid tail exhibited no frequency dependence due to their fast adsorption kinetics. The molecular exchanges between interface and bulk during compression and dilation were fast enough to compensate glycolipid concentration gradients at the interface which leads to low apparent interfacial elasticities. The interfacial elastic moduli of glycolipids with linear fatty acid tail were higher and increase monotonically with increasing frequency. Diffusion of these glycolipids from the interface into the bulk and vice versa was slower than with branched or monounsaturated fatty acid tail but still present at these dilatational frequencies. These surfactant concentration

fluctuations decrease with increasing dilatational frequency and cause the frequency dependence of the measured elastic moduli, which were thus not generated by intermolecular forces between a constant amount of surfactant molecules here.

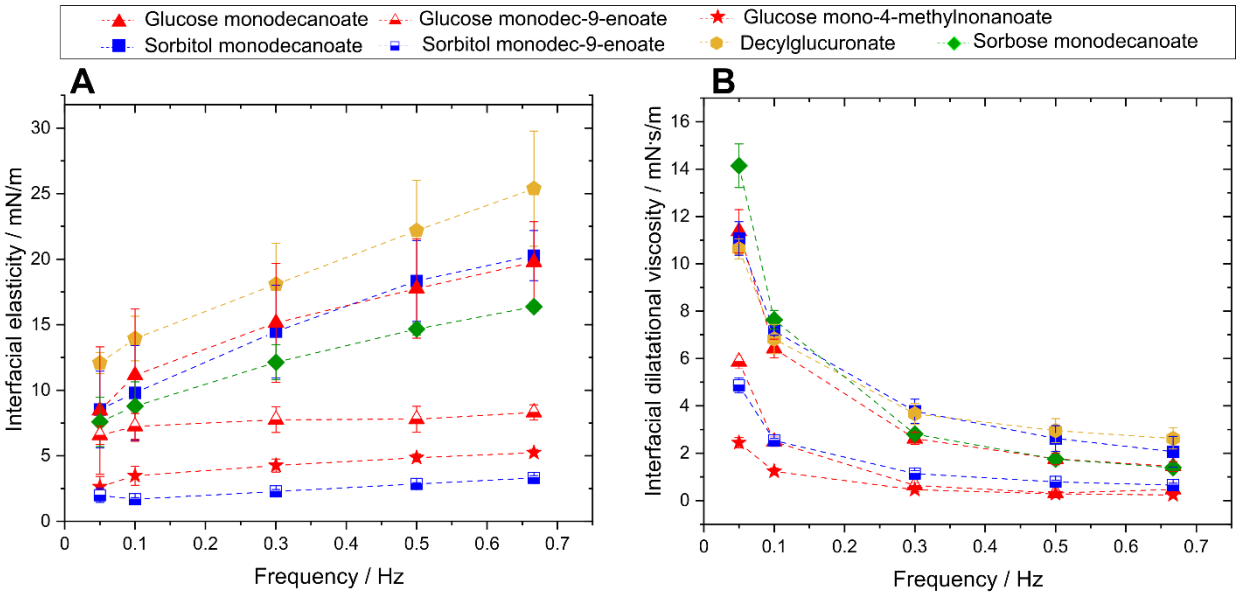

Figure 17. Interfacial rheological properties of the investigated glycolipids. A: Complex interfacial dilatational elasticity modulus and B: interfacial dilatational viscosity as a function of frequency. Unsaturated and branched glycolipids showed lower interfacial elasticity and interfacial viscosity than saturated, linear glycolipids.

Similar findings were observed for the dilatational interfacial viscosity (**Figure 17B**). Dynamic interfacial viscosity at 0.05 Hz was significantly higher for sorbose monodecanoate than for the other glycolipids. No significant differences were observed for glucose monodecanoate, sorbitol monodecanoate and decylglucuronate. Unsaturation in the fatty acid tail significantly lowered interfacial viscosity and branching resulted in the significantly lowest interfacial viscosity among the compared glycolipids. At frequencies of 0.1 Hz and higher, differences in the interfacial viscosity depending on the head groups were no longer significant while the glycolipids with unsaturated or branched hydrophobic tails had significantly lower interfacial viscosity over all frequencies tested. The tested glycolipids reached their equilibrium state of dilatational viscosity at the highest frequency tested.

4.3.3. Foam Stability

Foam stability is a key parameter when selecting suitable surfactants for the design of foamed commercial products (181).

The different investigated glycolipids were compared with regard to their ability to stabilize foam at glycolipid concentrations of twice the CMC value. Foams stabilized by glycolipids with a monounsaturated fatty acid tail were significantly less stable than those stabilized by glycolipids with saturated fatty acid tails (**Figure 18**). The decay time until reaching half of the initial foam height was 28 min for glucose monodec-9-enoate compared to 60 min for glucose monodecanoate and 25 min for sorbitol monodec-9-enoate compared to 30 min for sorbitol monodecanoate.

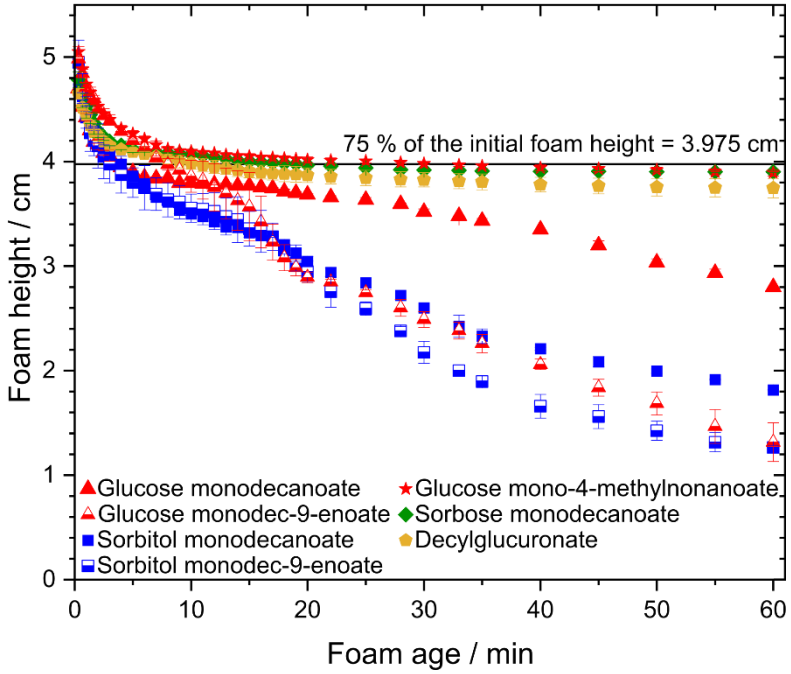

Figure 18. Foam stability of the investigated glycolipids, foam height vs. foam age. Foams stabilized by decylglucuronate, sorbose monodecanoate and glucose mono-4-methylnonanoate exhibited superior stability compared to the other glycolipids, while glycolipids with unsaturated fatty acid chain performed poorly.

In contrast, branched fatty acid tails significantly increased foam stability: the decay time until 75 % of the initial foam height was reached was 30 min for glucose mono-4-methylnonanoate compared to 4 min for glucose monodecanoate.

Foams stabilized by glucose mono-4-methyldecanoate, decylglucuronate and sorbose monodecanoate were most stable. Additionally, foam stability was higher with glucose monodecanoate than with sorbitol monodecanoate.

4.3.4. Bubble Size Distribution

Bubble size distribution was determined endoscopically. The initial bubble size distribution of foams stabilized by the seven glycolipids was quite similar except for decylglucuronate (**Appendix: Figure A4**).

After 600 s, a bimodal distribution was observed for foams stabilized by glycolipids with unsaturated fatty acid tails due to the formation of some huge bubbles (**Figure 19**). In contrast, monomodal distribution was detected directly after foam formation, as well as at a foam age of 10 min for foams stabilized by glycolipids including saturated fatty acid moieties.

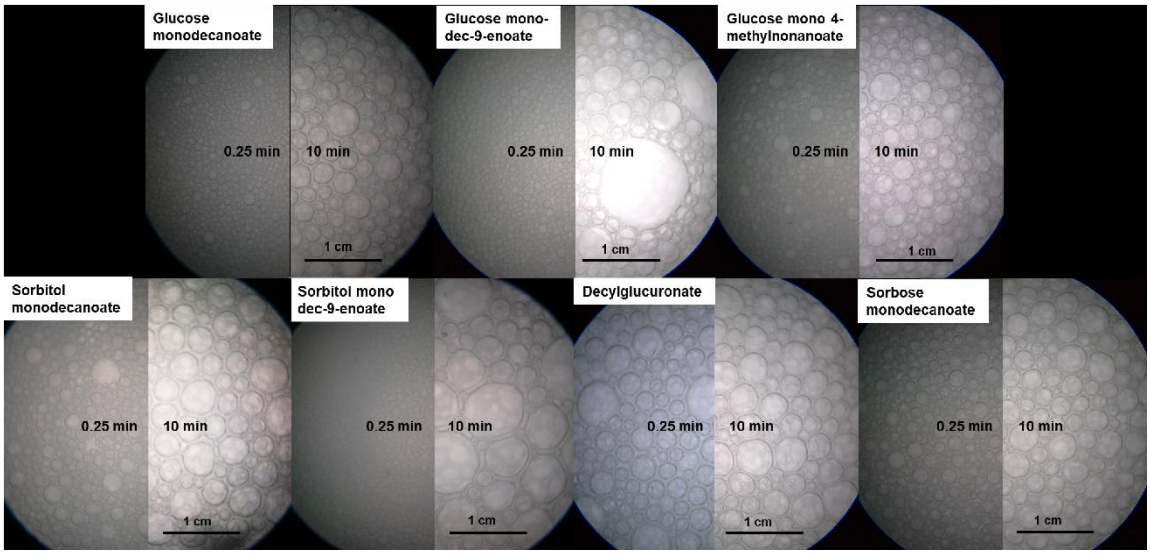

Figure 19. Endoscopic pictures of foams stabilized by glycolipids at 15 s and 600 s foam age. In foams stabilized by unsaturated glycolipids some huge bubbles occurred while bubble size of the other glycolipids is more homogenous.

Remarkably, the initial Sauter bubble diameter of the decylglucuronate foam was higher than for the other saturated glycolipids while the growth rates of the bubbles were similar (**Figure 20**).

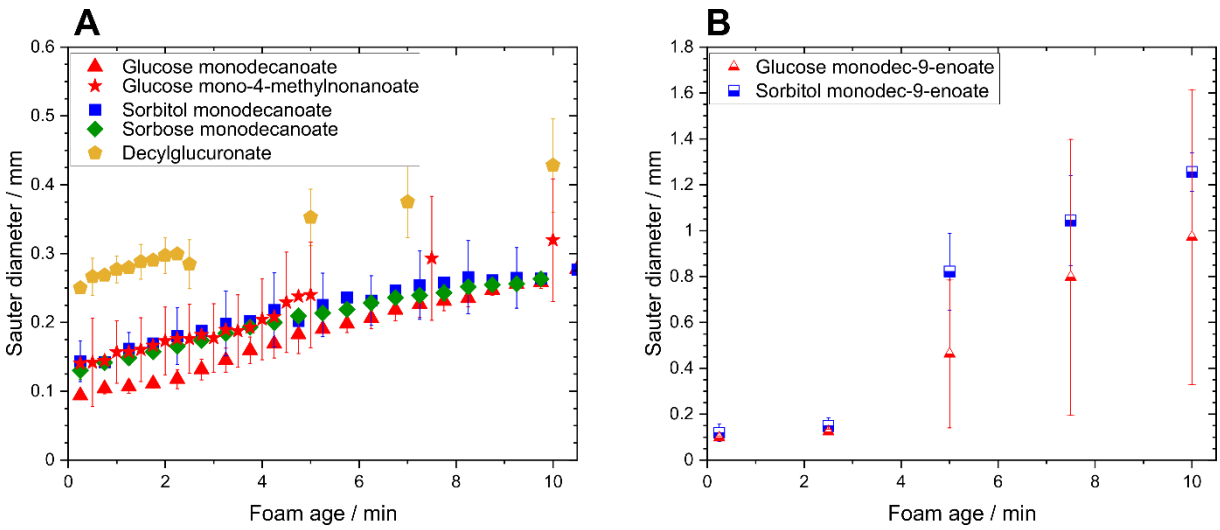

Figure 20. Sauter diameter of foams stabilized by different glycolipids as a function of foam age. A: Comparison of different head groups and influence of branching in the tail group. B: Impact of unsaturation in the hydrophobic tail group on Sauter diameter. Sauter diameter of foams stabilized by unsaturated glycolipids raised faster than those of the other glycolipids.

No differences in the Sauter bubble diameter of foams stabilized by glucose monodecanoate, sorbitol monodecanoate and sorbose decanoate foams were observed. Similar bubble size values were also found for the foam made from the glycolipid with the branched fatty acid moiety, glucose 4-methylnonanoate.

During the first 150 s of foam age, the Sauter bubble diameter of foams stabilized by saturated and unsaturated glycolipids did not differ, but did increase at higher foam ages faster for the foams of unsaturated glycolipids in contrast to those of saturated glycolipids. The coarsening rates of glucose monodecanoate, decylglucuronate, sorbitol monodecanoate and sorbose

monodecanoate were in a range of 90–200 μm²/s (**Appendix: Figure A5**). Branching had no significant effect on the coarsening rate, while unsaturated hydrophobic tails led to significantly higher coarsening rates of 2100 μm²/s for glucose monodec-9-enoate and 3400 μm²/s for sorbitol monodec-9-enoate.

4.3.5. Foam Gas Volume Fraction

The time evolution of gas volume fraction was determined using conductivity measurements. Although the initial gas volume fraction varies in a wide range (0.74–0.85), all foams reach essentially the same gas volume fraction of about 0.9 within 10 min. Remarkably, initial gas volume fraction of foams stabilized with decylglucuronate was significantly higher compared to the other glycolipids (**Figure 21A**). Branching in the fatty acid tail resulted in a lower value and slower raise of the gas volume fraction compared to non-branched glycolipids (**Figure 21A**). For foams made of sorbitol monodecanoate and glucose monodecanoate solutions, gas volume fraction increased faster than for the corresponding unsaturated glycolipids (**Figure 21B**).

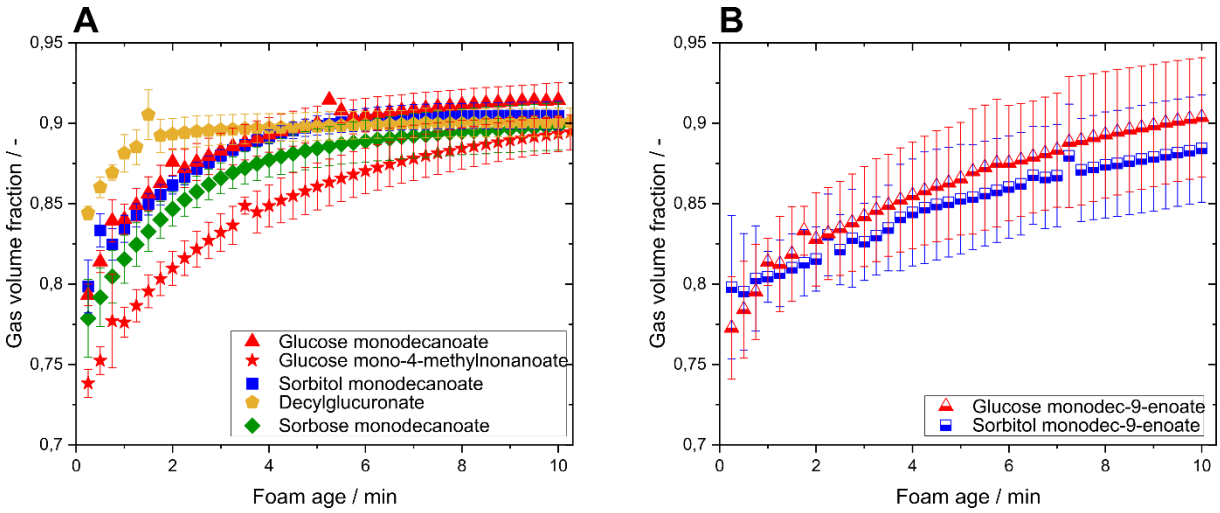

Figure 21. Gas volume fraction of the investigated glycolipids vs. foam age. A: Comparison of glycolipids with different head groups and influence of branching in the fatty acid tail. B: Comparison of saturated and unsaturated glycolipids.

4.3.6. Foam Elasticity

Foam elasticity characterizes the resistance of a foam against deformation. This is important for technological treatment during the production and transport of industrial foam products, for the texture and haptic sensation of food or cosmetic products.

Foam elasticity was characterized in terms of the shear modulus G_0, normalized by the bubbles' Laplace pressure given as the ratio of surface tension to Sauter bubble radius, at a gas volume fractions φ of 0.88 (**Figure 22**). Foams stabilized with unsaturated glycolipids could not be measured at gas volume fraction of 0.88 as foams were too fragile and collapsed under shear. The normalized shear modulus of foams stabilized with glucose mono-4-methyldecanoate was higher than for glucose monodecanoate. Foam made of decylglucuronate solution exhibited a significantly higher normalized shear modulus compared to the other head groups. However, differences in foam elasticity between sorbitol

monodecanoate and glycolipids containing another head group were not statistically significant.

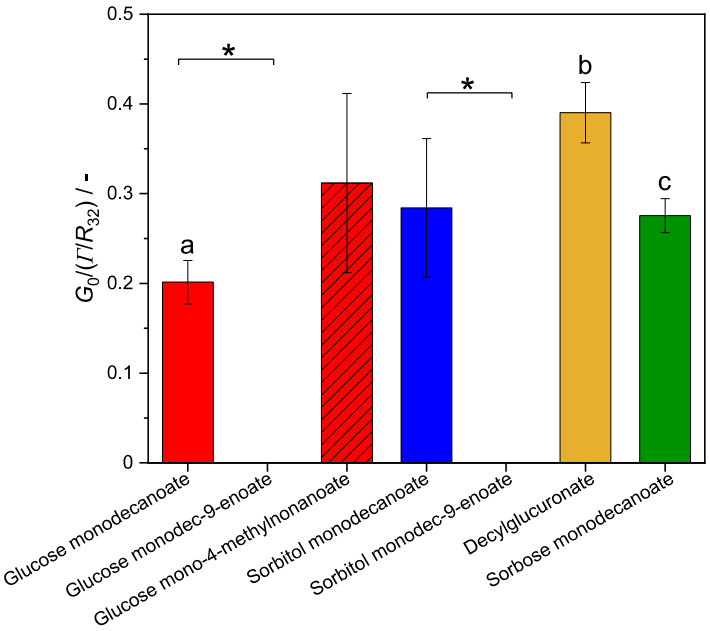

Figure 22. Shear modulus G_0 of glycolipid foams normalized by Laplace pressure Γ/R_{32} at a gas volume fraction of 0.88. * indicates statistically significant differences between tail groups. The letters a, b, and c indicate statistically significant differences between head groups. Unsaturation and branching in the hydrophobic fatty acid tail significantly influence the shear modulus G_0 while different head groups affect the shear modulus G_0 only slightly.

4.4. Discussion

In this study, seven glycolipids were enzymatically synthesized in order to determine the influence of different head and tail groups on interfacial and foam characteristics (**Figure 23**).

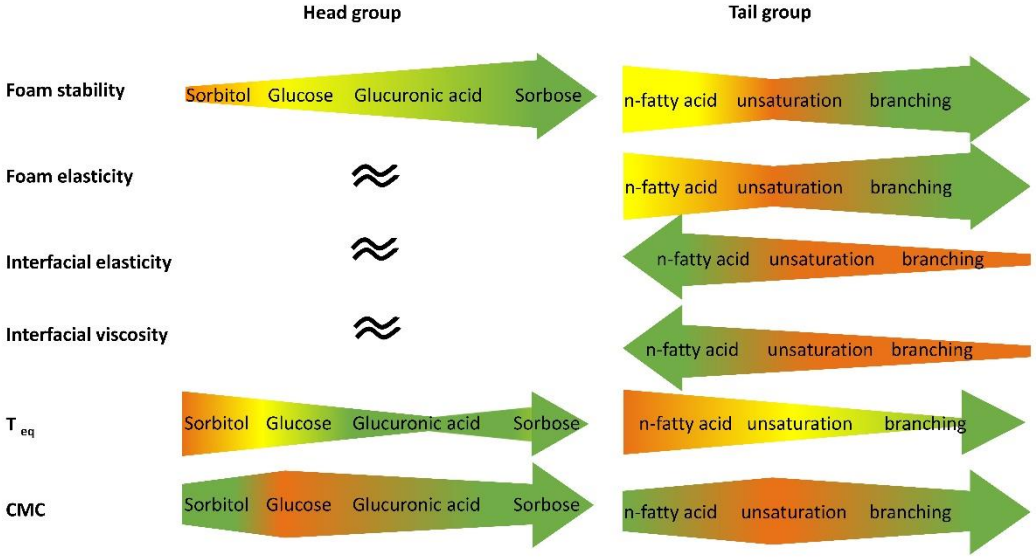

Figure 23. Summary of the results of the interfacial and foaming investigations. The width of the arrow indicates the size of the respective parameter. Green indicates higher interfacial activity/foam stabilizing property/elasticity. t_{eq} is the time when equilibrium interfacial tension is reached.

With regard to the tail group, results of the direct comparison of two saturated glycolipids, i.e., glucose and sorbitol monodecanoate, and their unsaturated derivatives, glucose and sorbitol mondec-9-enoate, indicate a strong influence of unsaturation in the hydrophobic fatty acid tail on interfacial and foaming properties. The unsaturated glycolipids had a CMC about two times higher than those with a saturated fatty acid tail. These results are in good accordance with those for unsaturated fatty acids, potassium fatty acids and sodium 10-undecenoate for which it was reported that each unsaturation doubles CMC (182,183). In the case of sophoroselipids and rhamnolipids, unsaturated fatty acid tails enhance the CMC value in contrast to saturated fatty acid tails (184,185).

Unsaturated glycolipids showed faster foam decay compared to saturated ones. Drainage is reported as the first instability effect to occur in foams (158). Foam aging can be classified into three different stages by analysis of the ratio of change in foam volume to change of drained volume of the surfactant solution over time (156). As long as this ratio is 1, foam volume decay is only drainage driven and no gas escapes from the foam. A transition to the second stage occurs when the ratio becomes greater than 1, because then additionally bubbles rupture and gas loss decreases foam volume. In the third stage, drainage has come to a halt and foam volume decay occurs due to bubble rupture only. For foams of glycolipids with unsaturated tail group, the classification of foam aging clearly indicates that foam decay of glucose monodec-9-enoate has a significantly shorter drainage-controlled stage 1 than glucose monodecanoate (**Figure 24**), even though the resistance of glucose monodec-9-enoate stabilized foam against drainage is higher (**Figure 21B**). The subordinate role of drainage for the foam instability mechanisms of glycolipids with unsaturation is confirmed by higher coarsening rates compared to saturated glycolipids and by the bimodality of the bubble size distribution at a foam age of 10 min.

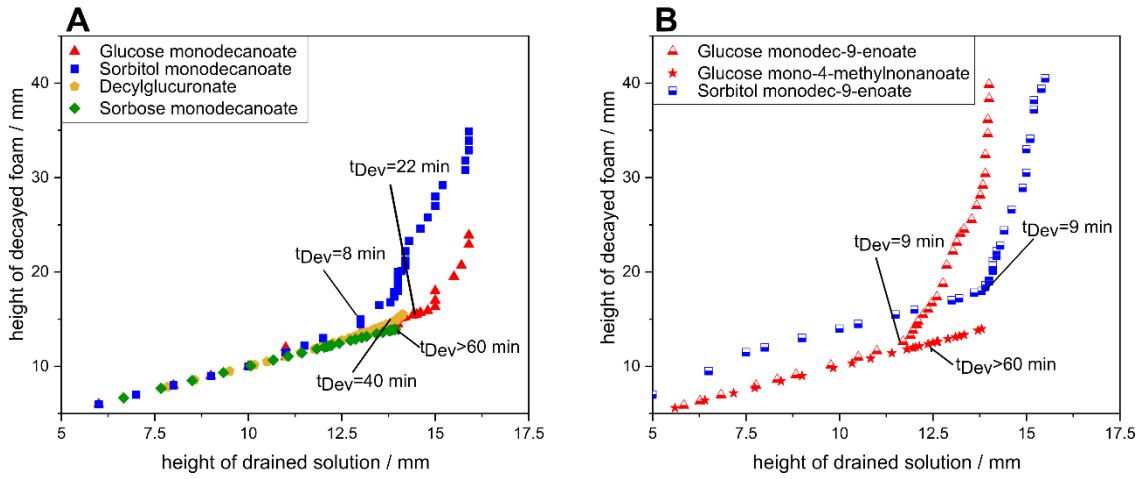

Figure 24. Classification of foam aging according to Lunkenheimer *et al.* (156). Ratio of changes in foam height to changes in solution height indicates drainage controlled decay when the ratio is 1. A: comparison of glycolipids with different head groups. B: comparison of unsaturation and branching in the hydrophobic tail group. t_{Dev}: time when drainage-controlled decay ends.

Interfacial elasticity, and to an even greater extent, interfacial viscosity, have already been reported to have stabilizing effects on foams (160,162,163). Since interfacial elasticity and interfacial viscosity of unsaturated glycolipids were also lowest, their interfacial rheological properties are likely a reason for the lower stability of corresponding foams. The lower interfacial elasticity and interfacial viscosity may cause the bimodality of the bubble size

distribution of the unsaturated glycolipids and not only coarsening but also coalescence occurred in these systems. Due to the unsaturation, van der Waals interactions between the tails are weaker than for the saturated glycolipids and this may be related to the higher CMC, lower interfacial elasticity and decreased foam stability.

Glucose mono-4-methylnonanoate was synthesized and investigated to study the influence of branching. The branched fatty acid tail led to a higher equilibrium interfacial tension compared to glucose *n*-monodecanoate while CMC values were similar. For lipopeptides, fatty acid tails containing iso-fatty acids have been reported to enhance biosurfactant surface activity and also for hydrocarbon surfactants with branched fatty acid tails stronger reduction of the interfacial tension were published (186–188). However, similar equilibrium interfacial tension and CMC values have been reported for branched and non-branched tridecanyl maltoside and octyl glucoside (168,189).

Glucose mono-4-methylnonanoate stabilized foams exhibited superior stability compared to non-branched glycolipids. Interfacial elasticity and interfacial viscosity of glucose mono-4-methylnonanoate were significantly lower than those of glucose monodecanoate. Therefore, interfacial rheological parameters can be excluded as reasons for the higher foam stability of the branched chain glycolipid, as well.

In the literature, contradictory results were reported on foaming properties of branched glycolipids: while Koeltow *et al.* described a branched tridecanyl maltoside having higher foaminess and foam stability than an *n*-tridecanyl maltoside (168), Waltermo *et al.* reported lower foam stability for branched octyl glucoside compared to a *n*-octyl glucoside (189). As the results of this study showed higher foam stability for the branched glucose monodecanoate compared to the unbranched glycolipid, it can be assumed that a minimal tail length is important for branching to enhance foam stability.

Drainage is slower in glucose mono-4-methylnonanoate stabilized foams as initial gas volume fraction was lower and a gas volume fraction of 0.9 was reached later compared to the non-branched glycolipids. Therefore, the retarded drainage is likely to be a reason for the higher foam stability with the branched glycolipid. This is supported by the classification of foam aging suggested by Lunkenheimer *et al.* (156). Branching in the fatty acid chain leads to an extended drainage-controlled stage 1 (**Figure 24**). Hence, for glucose mono-4-methylnonanoate, drainage is likely the dominating instability mechanism.

To investigate the influence of head groups, four glycolipids with different head groups were synthesized. Determined CMC values were between 0.7 mM to 1.5 mM.

With regard to interfacial properties, the CMC value for decylglucuronate was lower than that of glucose decanoate and also the adsorption of decylglucuronate at the interface was faster compared to glucose decanoate. This is likely due to the more hydrophobic character of decylglucuronate as the hydrophobic chain is not interrupted by a carbonyl group. Similar results were also observed for octylglucuronate compared to glucose octanoate; however, differences in the interfacial tension and interfacial rheology were reported (171). Contrarily, we did not observe significant differences in interfacial tension or in interfacial rheology for glucose monodecanoate and decylglucuronate. The differences between the results reported by Razafindralambro *et al.* (171) and the results of this study might be due to the longer tail length of decylglucuronate and glucose monodecanoate, and therefore, the differences in the

hydrophobic character of the molecules might be smaller and thus have less effect on interfacial properties.

Ducret *et al.* investigated CMC of glucose and sorbitol esters. For caprylates the CMC of the sorbitol ester was lower while for laurates the CMC for glucose esters was lower (152). In this study, the CMC of sorbitol decanoate was lower than that of glucose decanoate and therefore it can be assumed that for tail length up to C10 sorbitol esters have lower CMC values than glucose esters as the hydrophilic lipophilic balance decreases with increasing tail length. The measured values for the decanoates are in-between the values for caprylates and laurates (152).

Concerning foam characteristics, decylglucuronate and sorbose monodecanoate stabilized foams exhibited superior stability compared to the foams made from the glycolipids with the other head groups. However, interfacial elasticity and interfacial viscosity of decylglucuronate and sorbose monodecanoate were similar to those of glucose and sorbitol monodecanoate, and accordingly the differences in foam stability of these glycolipids cannot be explained by their interfacial rheological properties.

Comparison of the foam stability found for the different head groups of this study with literature values shows that the investigated glycolipids have a comparatively high potential for foam stabilization. While for the investigated glycolipids foam half-life is at least 30 min, a foam half-life of less than 10 min is reported for rhamnolipids at a concentration of 10 times CMC (190). For the biosurfactant surfactin, a residual foam volume after 20 min of 34 % was published at a concentration of 5 times the CMC (191,192). The synthetic surfactants methylestersulfonates (alkylchain length of 14–18 carbons) and polyoxyethylated dodecyl alcohol (3–9 ethoxy groups) show a half-life of no more than 3 min and 1.5 min at concentrations of 0.2 up to 5 times the CMC (157). However, the comparability of foaming experiments between different laboratories is limited, as the results can vary considerably with different methods and different gases used for foaming.

Comparing the effect of the different head groups on foam decay revealed that for the most stable foams with decylglucuronate and sorbose monodecanoate drainage was the mechanism controlling foam decay (**Figure 24**). The less stable foam made from sorbitol monodecanoate showed a shorter stage 1 than that made from glucose monodecanoate.

Although interfacial rheological properties alone do not explain foam stability and resistance against drainage sufficiently, foam stability as characterized by the foam height at 60 min foam age normalized to the initial foam height, correlated with the adsorption time required to reach equilibrium interfacial tension (**Figure 25**). Glycolipids characterized by shorter adsorption times exhibited higher foam stability. The glycolipids with a shorter adsorption time reach the interface faster and therefore stabilize the bubbles more efficiently. This is supported by the findings of Petkova *et al.*, who determined a correlation between dynamic interfacial tension and foaminess for non-ionic surfactants (193).

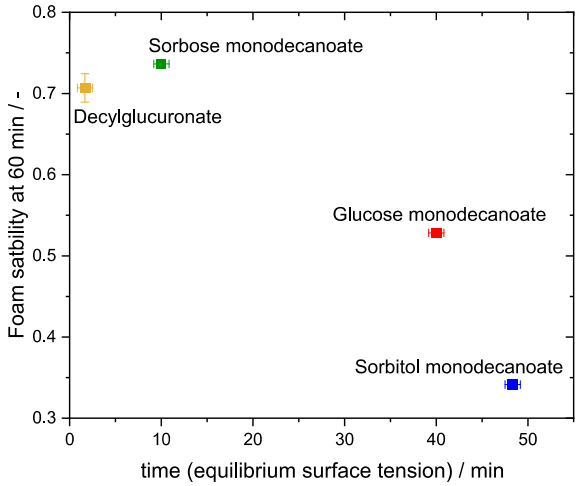

Figure 25. Correlation of foam stability at 60 min with time to reach equilibrium surface tension. Foams stabilized by glycolipids with a faster adsorption at the interface exhibit higher foam stability. Foam stability = $\frac{\text{Foam height at 60 min}}{\text{Initial foam height}}$.

In general, interfaces in foams of non-ionic surfactants are predominantly stabilized by repulsion forces between surfactant molecules (193). In the case of glycolipids electrostatic repulsion contributes to repulsion forces due to the hydration of the head group (164,165,194,195). Aldoses and ketoses exhibit different degrees of hydration (196). Glucose is an aldose while sorbose is a ketose, glucuronic acid is an uronic acid and sorbitol is an alditol. Therefore, despite similar interfacial rheology the differences in dynamic interfacial tension and foaming properties between glucose monodecanoate, sorbose monodecanoate and sorbitol monodecanoate are likely due to their different hydration which causes differences in the repulsion forces between the surfactant molecules and consequently also in the foam films. However, the interactions between sugar head groups at interfaces are not well understood yet.

In summary, all investigated glycolipids exhibited promising foam stability compared to different synthetic surfactants as well as biosurfactants described in the literature (157,190–192). Nevertheless, the results of this study indicate that ketoses are more suitable head groups for glycolipids than aldoses or alditols with respect to foam stabilizing properties. Furthermore, our results suggest a preference of branched fatty acid groups over unbranched or unsaturated fatty acid groups for foam applications. Sorbose monodecanoate yields the highest potential among the investigated glycolipids for application as foaming agent, because its foam performs best with respect to volume stability over time, rate of bubble size and gas volume fraction change. It finally also provides a high foam elasticity at a relatively low surfactant concentration of 0.2 %.

4.5. Conclusions

The aim of this study was to investigate the structure–function relationship of seven enzymatically synthetized glycolipids with regard to their interfacial and foaming properties. Hereby, four different head groups, glucose, glucuronic acid, sorbose and sorbitol were evaluated, as well as unsaturation and branching in the C10 fatty acid tail.

Unsaturation in the fatty acid tail resulted in increased CMC and reduced interfacial elasticity, interfacial viscosity and foam stability. Branching also reduced interfacial elasticity and interfacial viscosity but increased foam stability. Glycolipids with different head groups showed only insignificant differences in interfacial rheological properties as well as foam elasticity. However, decylglucuronate and sorbose monodecanoate showed superior foam stability over glucose monodecanoate and sorbitol monodecanoate. These results indicate that among the tested sugar(-derivatives), ketoses and uronic acids have a higher potential as glycolipid head group for foaming applications than aldoses or alditols. Adsorption time at the interface was identified as crucial parameter for foam stability.

Consequently, this study reveals that both the head group, despite its minor influence on interfacial properties, and the functional groups in the fatty acid are crucial factors for foam stability. In a subsequent study, gas permeability, film thickness, film contact angle and surface forces of individual foam lamellae should be investigated in order to obtain more profound insights into the processes at the interfaces. Furthermore, technical characterization of the investigated glycolipids in terms of emulsification, greasing power and skin compatibility should be addressed.

5. Summary and Final Conclusion

Glycolipids present a promising alternative to petrochemically-derived surfactants. They can be synthetized enzymatically in deep eutectic solvents which are a sustainable solvent alternative to organic solvents. However, quantitative analysis of glycolipid synthesis in DES are still lacking and available quantification methods are limitied to indirect quantification. Therefore, the aim of this study was first, to develop a direct quantification method for glycolipid production and to evaluate the extraction of glycolipids from hydrophilic DES. The developed HPLC-ELSD method allowed for sensitive and direct glycolipid quantification with a quantification limit of 1.4 µM. In glycolipid extraction from two hydrophilic DES, ethyl acetate revealed to be a superior extraction solvent compared to dimethyl carbonate and the harmful chloroform. Glycolipids show higher extractability in the more polar solvents ethyl acetate and dimethlycarbonate than in the less polar solvent chloroform as they are polar, amphiphilic molecules. However, the results indicate that the investigated glycolipid, glucose monodecanoate is less polar than dimethylacarbonate and therefore better extractable with less polar ethyl acetate. Supercritical CO_2 represents another possible solvent for glycolipid extraction from DES and should be investigated in future studies.

To characterize enzymatic glycolipid production in hydrophilic DES, it was intended to evaluate different reaction parameters that were identified as crucial in organic solvents. The investigated hydrophilic DES consisting of choline chloride and urea or glucose exhibit high viscosities compared to water or organic solvents. However, an external mass transfer limitation can be excluded as sufficient mixing was reached at agitation rates of 60 rpm and higher. Instead, fatty acid availability was identified as a limiting factor in hydrophilic DES for the first time and an inhibiting effect of high fatty acid concentrations was shown. Initial reaction velocities were highest at a fatty acid concentration of 0.5 M for both DES while higher fatty acid concentrations resulted in decreased reaction rates. Fatty acid-DES emulsions showed a bimodal droplet size distribution. By ultrasonic treatment, bimodality was reduced and the droplet size distribution was shifted towards smaller droplet sizes resulting in increased reaction rates. Therefore, ultrasonic treatment was presented as strategy to improve fatty acid distribution whereby glycolipid yields were increased four times compared to synthesis in untreated fatty acid-DES emulsions. Nevertheless, the polarity of DES also influenced glycolipid synthesis as reaction rates and yields, both, were higher in the less polar choline chloride: urea-DES compared to the more polar choline chloride: glucose-DES.

In future studies, further strategies for physical pretreatment of fatty acid-DES emulsions, like homogenization, should be evaluated in order to identify the most efficient treatment method.

Fatty acid accessibility was identified as a limiting factor in hydrophilic DES, while at the same time high fatty acid concentrations led to an inhibition of the lipase. Therefore, the applicability of a hydrophobic (-)-menthol: decanoic acid-DES was addressed for the first time. In this DES, the fatty acid is already part of the medium and thus well distributed while inhibiting effects of the fatty acid might be reduced due to the DES structure. Yield and reaction rates were significantly higher in the hydrophobic DES compared to the hydrophilic DES used previously. By increasing the amount of glucose, the yield could be increased even further to 1000 times that of the hydrophilic choline chloride: urea DES. Moreover, both free and vinylated fatty acids can be used in this reaction medium, so that the activation step of the

fatty acid is no longer mandatory. However, monitoring of the glycolipid production and water concentration in esterification reaction revealed that the water release during esterification presents a limiting factor which is absent in transesterification. Furthermore, the applied lipase revealed to be highly stable in the (-)-menthol: decanoic acid-DES. A reuse of the enzyme over at least five cycles without loss of activity was achieved. Therefore, the problem of limited fatty acid accessibility was successfully solved by introducing a hydrophobic DES in enzymatic glycolipid synthesis, and no inhibitory effect of the fatty acid was found in this novel system.

To further promote glycolipid synthesis in DES, future research should focus on the applicability of DES with intermediate polarity and further hydrophobic DES in order to identify DES that allow for highly efficient (trans-)esterification.

Finally, it was aimed to investigate the structure-function relationship of glycolipids as not only the sustainable synthesis qualifies them as an alternative to petrochemical surfactants but also the properties of the glycolipids need to be competitive with petrochemical surfactants in order to represent an actual alternative. Hereby different head groups and tail groups were compared. Interfacial elasticity and interfacial viscosity did not show correlation with foam stability even though these parameters are reported as stabilizing mechanisms in foam aging for other surfactant classes. Instead, dynamic interfacial tension showed correlation with foam stability as glycolipids with a shorter adsorption time at the interface exhibited higher foam stabilizing abilities than those with longer adsorption times. Glycolipids with sorbose and glucuronic acid head group showed superior performance compared to those with glucose or sorbitol head groups in regard to higher foam stabilizing ability, faster adsorption time and lower CMC value. Different hydration of the head groups is most likely the reason for their different performances at interfaces. Glycolipids with an unsaturated fatty acid group revealed high CMC values, low foam stability and a bimodal bubble size distribution, as well as low interfacial elasticity and viscosity indicating that the intermolecular forces are weakened. An increased adsorption rate and increased foam stability could be achieved by introducing a branching in the fatty acid tail, as well as increased foam elasticity. Hence, this thesis demonstrated for the first time that branching in the hydrophobic tail leads to increased interfacial activity not only in hydrocarbon surfactants but also in glycolipids.

Since the investigated glycolipids exhibit excellent interfacial and foam stabilizing activity, their emulsification and detergent properties should be addressed in subsequent studies.

In this thesis, the influence of different reaction parameters on glycolipid synthesis in DES was evaluated. Similarities in the influence of fatty acid concentration, sugar loading and solvent hydrophobicity on lipase-catalyzed glycolipid synthesis between organic solvents, IL and DES were found. High fatty acid concentrations exhibited inhibiting effects also in hydrophilic DES. Supersaturated sugar solutions were reported to result in highest glycolipid yields in organic solvents and IL. This was shown, in this thesis, also for the synthesis in DES. Nevertheless, the suitability of a hydrophobic DES for lipophilization of sugars was demonstrated for the first time. The comparison between the two investigated hydrophilic DES and the hydrophobic (-)-menthol: decanoic acid DES revealed that DES with higher hydrophobicity allow for a more efficient synthesis and therefore highlighted the influence of solvent polarity on enzymatic glycolipids synthesis in DES for the first time. Hence, this thesis

provides a broad understanding of decisive parameters for lipase-catalyzed glycolipid synthesis in deep eutectic solvents.

Furthermore, structure-function relationship of tailor-made glycolipids was addressed. Hereby it could be clearly demonstrated that the head group significantly influences foaming properties despite its minor influence on the surface tension. Thus, not only the fatty acid group must be considered in designing glycolipids, but also the head group was proven to have a high value for the modification of properties. These results provide guidance for the selection of suitable combinations for head and tail groups for tailor-made glycolipids.

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List of Abbreviations

ChCl:Glc	Chloline chloride: glucose -DES
ChCl:U	Choline chloride: urea -DES
CMC	Critical micelle concentration
DES	Deep eutectic solvents
DMC	Dimethyl carbonate
ELSD	Evaporative light scattering detector
EtAc	Ethyl acetate
HPLC	High performance liquid chromatography
iCalB	immobilized *Candida antarctica* lipase B
IL	Ionic liquids
VD	Vinyl decanoate

Appendix

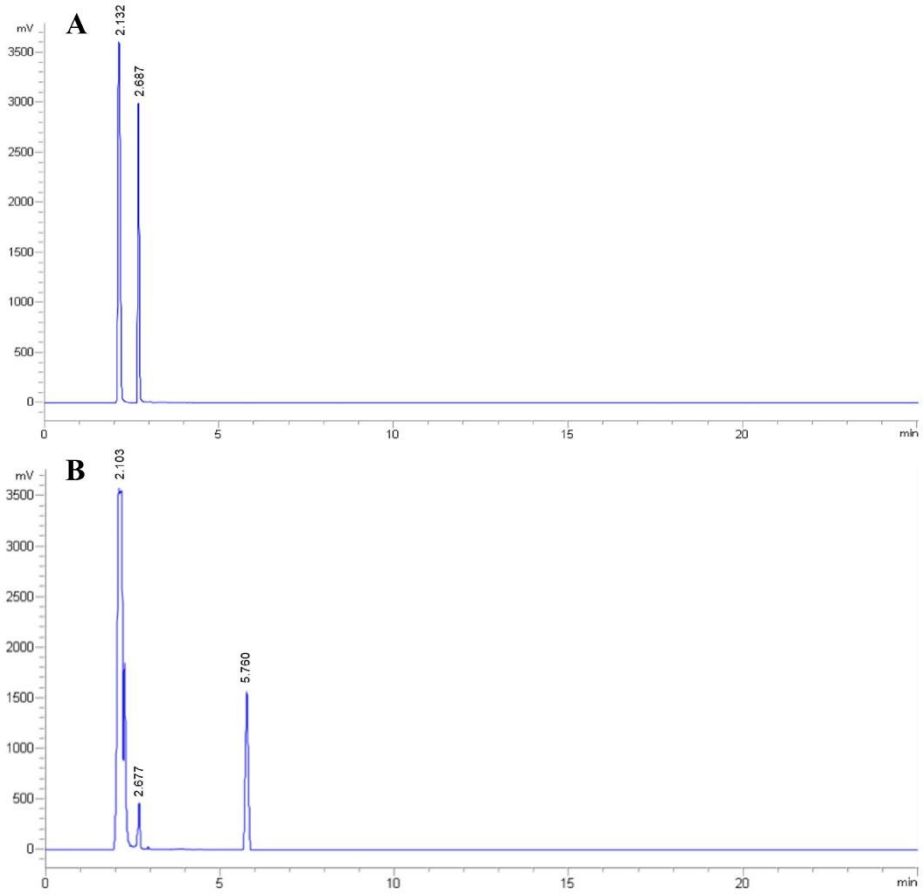

Figure A1. Chromatograms of HPLC-ELSD separation of extracts from glycolipid synthesis. A shows the separation of an extract of ChCl:U with added glucose monodecanoate, at 2.1 min urea elutes and at 2.7 min glucose monodecanoate elutes. B shows the chromatogram of an extract from a 24 h synthesis of glucose monodecanoate in ChCl:U, the peak at 2.1 min presents urea, at 2.7 min glucose monodecanoate and at 5.7 min decanoic acid.

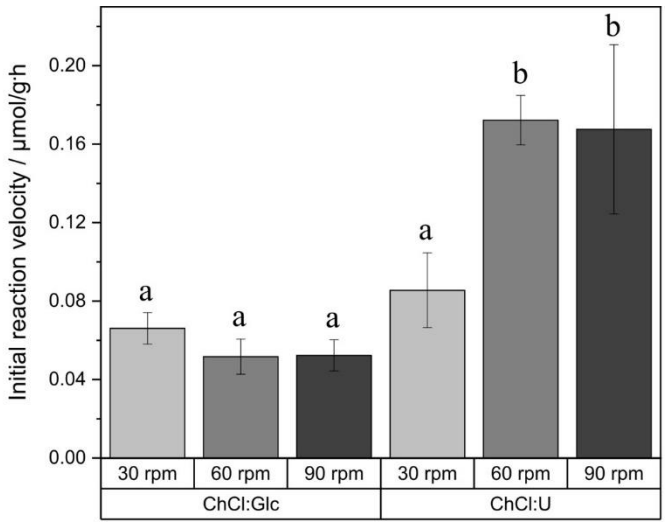

Figure A2. Initial reaction velocity of sonicated samples in relation to the agitation rate. Reaction conditions: 0.5 M vinyl decanoate, 50 °C. a,b, shows statistically significant differences. ChCl:Glc (ChCl:Glc:H_2O, 5:2:5, n:n:n) and ChCl:U (ChCl:U, 1:2, 5 % water).

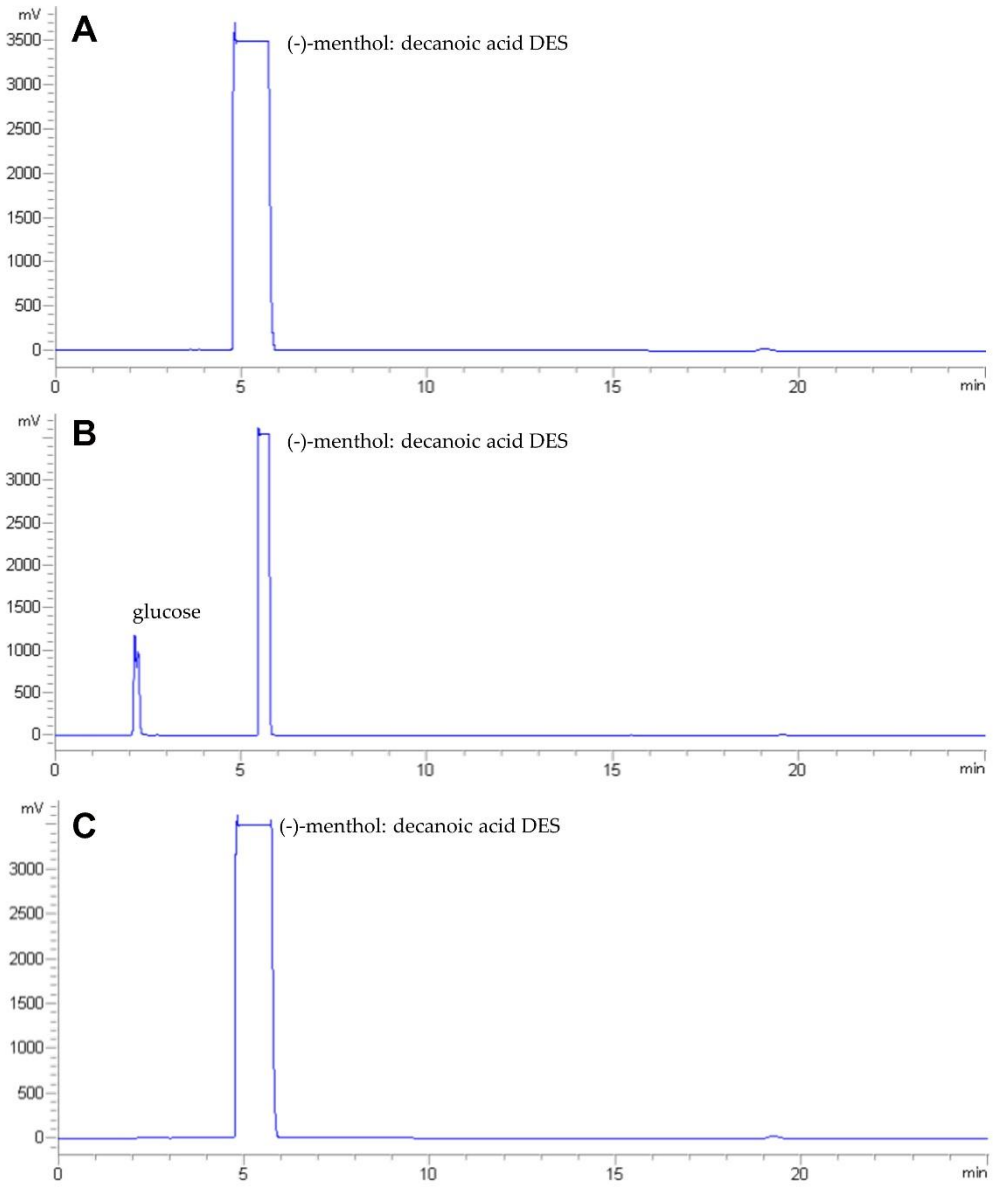

Figure A3. HPLC-ELSD-Chromatograms of pure DES and negative controls of the synthesis. Chromatograms clearly indicate no product or side product formation occurs in the negative controls. A: pure (-)-menthol: decanoic acid DES diluted with ethyl acetate, B: negative control without addition of enzyme at 120 h reaction time, C: negative control without addition of glucose at 120 h reaction time.

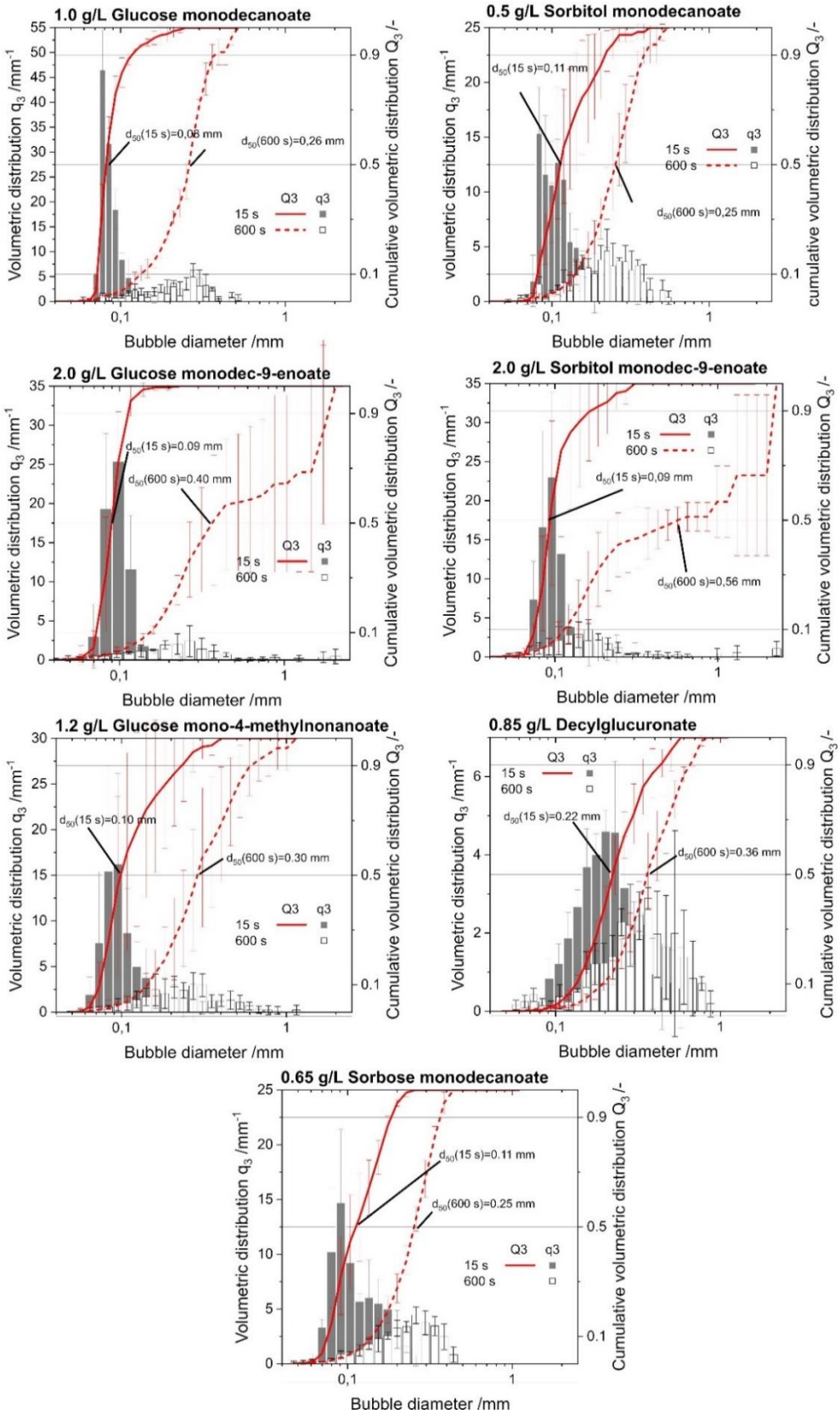

Figure A4. Bubble size distributions of the glycolipids at 15 s and 600 s foam age. Volumetric density histogram, as well as cumulative volumetric bubble size distribution are shown: Glucose monodecanoate; Glucose monodec-9-enoate; Glucose mono-4-methylnonanoate. Sorbitol monodecanoate. Sorbitol monodec-9-enoate. Decylglucuronate. Sorbose monodecanoate.

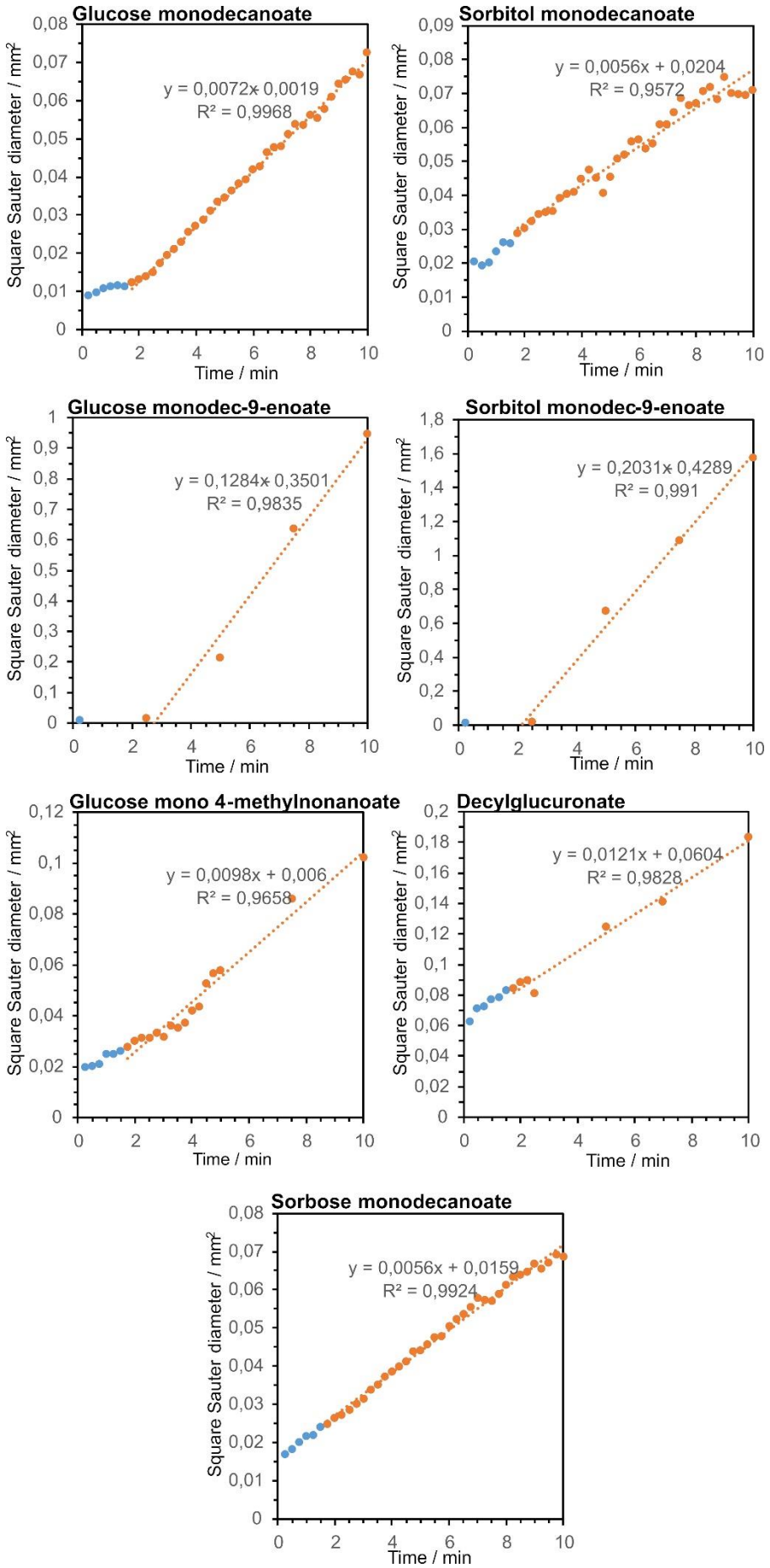

Figure A5. Square sauter diameter versus time plots for foams stabilized by glycolipids