Enzymatic Synthesis of Glycolipid Surfactants

Utilization of Sustainable Media and Substrates

zur Erlangung des akademischen Grades eines
DOKTORS DER INGENIEURWISSENSCHAFTEN (DR.-ING.)

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: 29. März 2019

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"Sage es mir, und ich vergesse es;

zeige es mir, und ich erinnere mich;

lass es mich tun, und ich behalte es."

Konfuzius

Danksagung

Hiermit möchte ich mich bei all jenen bedanken, die in jeglicher Weise am Gelingen dieser Arbeit beigetragen und mich währenddessen unterstützt haben.

Herrn Prof. Christoph Syldatk für die Möglichkeit, meine Doktorarbeit in seiner Arbeitsgruppe durchzuführen. Danke für die Konzeption dieser Arbeit, die konstruktiven wissenschaftlichen Diskussionen und der Unterstützung während meiner Zeit am Tebi.

Herrn Prof. Steffen Rupp für die erfolgreiche Kooperation und die freundliche Übernahme des Referates.

Einen großen Dank gebührt meinen Studenten und Studentinnen Tatjana, Fabian, Jochen, Alexandre, Julian, Jennifer und Maria. Danke für die tolle Zusammenarbeit und euren großartigen Einsatz in- und außerhalb des Labors.

Vielen Dank an meine Kollegen Marius, Johannes, Judith, Julia, Sarah & Christin, Florian, Xiujuan, Fei, Vanessa, Rebecca, Caroline, Marcus, Ulrike, Habibu, Anke und Jens R für die gute und lustige Arbeitsatmosphäre, netten Gesprächen und Tipps. Danke an Laura, Michaela, Delphine und Pascal für die Hilfe bei der Analytik und den Fermentationen, Danke an Sandra für die Einarbeitung im Labor. Ein großer Dank geht an Katrin für Ihr Feedback, das Reviewen vieler Texte und die gute Zusammenarbeit im gemeinsamen Projekt. André (fürs „Praktikum“), Olga (für Instagram), Alba (für ihr Englisch), Olli und Jens (auch für die Fachgespräche), Teresa (fürs Babysitten) und besonders Stefan (als Büronachbar warst du Klasse) ein großes Dankeschön für die unvergessliche Zeit hier in Karlsruhe.

Einen großen Dank an Frank Kirschhöfer, Gerald Brenner-Weiß und Ihren Kollegen für die Hilfe bei der Massenspektrometrie und den Tagen am CN, Miriam, Claudia Muhle-Goll und Prof. Luy für die vielen NMRs und der Unterstützung beim Auswerten, Ekkehard Hiller, Michael Günther und Fabian Haitz für die TLC-MALDIs, Thomas Hahn und Susanne Zibek für das Bereitstellen der Lignocellulosefraktion, Warc Wittner und Volker Gaukel für die Sprühtrocknung, Tim Siebert für die Bestimmung der Wasseraktivität meiner DES sowie Lydia Schütz für die Messungen am Rheometer, Lutz Großmann und Prof. Weiss für die Messung der Wasseraktivität von Honig und Agavendicksaft, Marius Henkel und Prof. Hausmann für die HPTLC Messungen, sowie Alexander Wilsdorf und Prof. Krastev für die Möglichkeit zur Bestimmung der Tensideigenschaften. Ohne euch wäre diese Arbeit nur halb so gut und halb so lang.

Merci beaucoup an Erwann Durand und Pierre Villeneuve dafür, dass Ihr mich für einen Monat in eurer Arbeitsgruppe in Montpellier aufgenommen habt und für die weitere Zusammenarbeit. Ich habe in der Zeit bei euch wahnsinnig viel gelernt. Auch ein Dankeschön an die restlichen Mitarbeiter und all jene die ich in Montpellier kennen gelernt habe für die schöne Zeit.

Ich danke außerdem der Baden-Württemberg Stiftung für die Förderung innerhalb des Graduiertenkollegs BBW ForWerts und dem KHYS für die Förderung meines Aufenthaltes in Montpellier.

Ich möchte mich bei meinen Eltern für ihre Unterstützung und Geduld in den vielen Jahren an der Uni danken, sowie meinen Freunden für schöne Diskussionen und benötigte Ablenkung.

Aber der größte Dank gilt meiner Frau Katja für ihre Unterstützung und Aufmunterungen sowie meinen Sohn Frederick (und auch meinen Sohn Tristan, der mich in den letzten Zügen des Schreibens wachgehalten hat) für sein Lachen, seine Unbekümmertheit und der Erkenntnis, dass man auch ohne viel Schlaf und Kaffee promovieren kann.

Preamble

Parts of this thesis are based on peer reviewed research articles. All articles have been drafted during this work and describe the major results of the enzymatic synthesis of glycolipid biosurfactants in non-conventional media and substrates. Chapters based on previous published work are indicated as such at the beginning of the chapter. The text of these chapters is partly identical to the content of the publications. Layout, citation style, figures and formating have been modified and adjusted to the style of this dissertation.

Publications and Presentations

Original Research Papers

Sascha Siebenhaller, Claudia Muhle-Goll, Burkhard Luy, Frank Kirschhöfer, Gerald Brenner-Weiss, Ekkehard Hiller, Michael Günther, Steffen Rupp, Susanne Zibek and Christoph Syldatk

Sustainable Enzymatic Synthesis of Glycolipids in a Deep Eutectic Solvent System

Journal of Molecular Catalysis B: Enzymatic (2016); Volume 133, page 281-287

DOI: 10.1016/j.molcatb.2017.01.015

Sascha Siebenhaller, Tatjana Hajek, Claudia Muhle-Goll, Miriam Himmelsbach, Burkhard Luy, Frank Kirschhöfer, Gerald Brenner-Weiß, Thomas Hahn, Susanne Zibek, Christoph Syldatk

Beechwood Carbohydrates for Enzymatic Synthesis of Sustainable Glycolipids

Bioresources and Bioprocessing (2017); Volume 4, article 25

DOI: 10.1186/s40643-017-0155-7

Sascha Siebenhaller, Jennifer Kirchhoff, Frank Kirschhöfer, Gerald Brenner-Weiß, Claudia Muhle-Goll, Burkhard Luy, Katrin Ochsenreither and Christoph Syldatk

Lipase-Catalyzed Synthesis of Sugar Esters in Honey and Agave Syrup

Frontiers in Chemistry (2018); Volume 6, article 24

DOI: 10.3389/fchem.2018.00024

Sascha Siebenhaller, Julian Gentes, Alba Infantes, Claudia Muhle-Goll, Frank Kirschhöfer, Gerald Brenner-Weiß, Fabian Haitz, Thomas Hahn, Susanne Zibek, Christoph Syldatk and Katrin Ochsenreither

Integrated Process for the Enzymatic Production of Fatty Acid Sugar Esters Completely Based on Lignocellulosic Substrates

Frontiers in Chemistry (2018); Volume 6, article 421

DOI: 10.3389/fchem.2018.00421

Magdalena Pätzold, **Sascha Siebenhaller**, Selin Kara, Andreas Liese, Christoph Syldatk, Dirk Holtmann

Deep Eutectic Solvents as Efficient Solvents in Biocatalysis

Trends in Biotechnology

DOI: 10.1016/j.tibtech.2019.03.007

Book Chapter

Sascha Siebenhaller, Jens Grüninger and Christoph Syldatk

Enzymatic Synthesis of Glycolipid Surfactants (Chapter 13).

In *"Lipid Modification by Enzymes and Engineered Microbes"*, 2018

Academic Press and AOCS Press

ISBN: 9780128131671

DOI: 10.1016/B978-0-12-813167-1.00013-X

Poster Presentations

Sascha Siebenhaller, Christoph Syldatk

Enzymatische Synthese von maßgeschneiderten Biotensiden auf Basis von Lignocellulose

1. Internationaler Bioökonomie-Kongress Baden-Württemberg (2014), Stuttgart, Germany

Sascha Siebenhaller, Christoph Syldatk

Enzymatic synthesis of tailor-made glycolipids in deep eutectic solvents

Biocat2016; 8th International Congress on Biocatalysis (2016), Hamburg, Germany

Sascha Siebenhaller, Christoph Syldatk

Organosolv-Sugars for the Enzymatic Synthesis of Biosurfactants in Deep Eutectic Solvents

BNCM 2017; 5th Conference on Biocatalysis on Non-Conventional Media (2017), Rostock, Germany

Sascha Siebenhaller, Christoph Syldatk

Enzymatic Glycolipid Synthesis in Deep Eutectic Solvents with Organosolv-Sugars

15th Euro Fed Lipid Congress; Oil, Fats and Lipids: New Technologies and Applications for a Healthier Life (2017), Uppsala, Sweden

Sascha Siebenhaller, Christoph Syldatk

Enzymatically Synthesized Glycolipids in Organosolv-Sugar based Deep Eutectic Solvents and their Quantification

2. Internationaler Bioökonomie-Kongress Baden-Württemberg (2017), Hohenheim, Germany

Conference Talks

Sascha Siebenhaller, Tatjana Hajek, Susanne Zibek und Christoph Syldatk

Enzymatische Synthese von Biotensiden aus der Cellulosefraktion des Organosolvprozesses

ProcessNet-Jahrestagung und 32. DECHEMA-Jahrestagung der Biotechnologen (2016), Aachen, Germany

Abstract available in: Chem. Ing. Tech. 2016, 88, No. 9; ISSN 0009-286 X

DOI:10.1002/cite.201650171

Abstract

Surfactants are a diverse group of amphiphilic molecules which can reduce the surface tension and act as detergents, emulsifiers or wetting agents. Therefore, they are an important economic factor and are produced in million tons per year.

Surfactants are a suitable example for an incipient mindset change in our economy. Whereas surfactants used to be produced chemically from crude oil, today renewable components are often used and an ever increasing number of environmentally friendly enzymatic processes are being established for their production.

This thesis is embedded in the BBW ForWerts Graduate Program of the State of Baden-Württemberg (Bioeconomy Baden-Württemberg: Exploring Innovative Value Chains) and the main goal is the enzymatic synthesis of novel and sustainable surfactants, mainly glycolipids, from renewable substrates in environmental friendly media.

Glycolipids are surfactants with a polar sugar-head and a hydrophobic tail. They show various beneficial characteristics like low toxicity, a good biodegradability and a high surface activity, mostly combined with a low CMC value. They can be produced either chemically, or by means of an eco-friendly enzymatic reaction. For this, a nearly water-free solvent is needed to shift the equilibrium towards acylation. Various sugars are possible as substrates, but they are poorly soluble in the needed water-free nonpolar organic solvents. Therefore, an ecologically-friendly deep eutectic solvent (DES) system for the enzymatic biosurfactants synthesis was established in this thesis (chapter 2). Several DES were formed by mixing and heating the ammonium salt choline chloride and a sugar in a suitable molar ratio. The so formed DES acts simultaneously as an environmentally-friendly reaction media and substrate for a lipase catalyzed synthesis of glycolipids. In total, five sugars, three sugar alcohols and one anhydrosugar were used in combination with up to nine fatty acids. The successful formation of surfactants was proven by thin-layer chromatography, mass- and NMR analysis. Besides that, for the first time an anhydrosugar-based DES was formed and a successful enzymatic synthesis was performed in it.

In chapter 3, the time-depending formation and reaction yields of arabinose laurate and di-laurate, which acts here as a reference glycolipid, were determined. First, the best suitable ratio of choline chloride and

arabinose and secondly, the optimal water content, along with the water activity for the DES reaction media were determined in order to optimize the (future) enzymatic arabinose laurate synthesis. The third parameter investigated was the synthesis duration. To determine the time-dependent formation of arabinose laurate and di-laurate, several reactions were performed and analyzed via high performance thin-layer chromatography. Furthermore, the critical micelle concentration of arabinose laurate as well as its potential to reduce the surface tension of water were determined. It was observed that the addition of small amounts of water (approx. 8 %) can more than double the yield of arabinose laurate (up to 49.6 % after 48 h); arabinose di-laurate was only formed if no additional water is added. With higher water concentrations, the yield slightly decreases due hydrolysis of the products. The majority of the products were synthesized within 24 h, longer reaction times have only a small influence on the yields. Besides, the ratio of the DES components had only minor impact on the yields. Arabinose laurate show promising properties: it can lower the surface tension of water drastically to 23 mN/m, but the CMC value of 530 mg/L is much higher than for other known glycolipids.

Chapter 4 deals with the usage of sugars from beech wood as a sustainable source for the glycolipids sugar moiety. The carbohydrates were obtained by an acid-catalyzed organosolv pretreatment process, in which beech wood was converted in a fiber fraction that was subsequently hydrolyzed to obtain the monosaccharides. After purification and drying, this glucose- and xylose-rich fraction was used to create a deep eutectic solvent. By using this already established reaction system, the sugars act both as solvent and substrate for the lipase-catalyzed reaction at the same time. Finally, the successful synthesis of glycolipids with a polar head made of a sustainable glucose or xylose molecule were confirmed by ESI-Q-ToF mass spectrometry and multidimensional NMR experiments. Moreover, conversion yields of 4.8 % were determined by LC-MS/MS.

To produce sugar esters completely based on sustainable resources, a source for the fatty acids was needed. To avoid the usage of plant oils, the unpurified monosaccharide solution was used as carbon source for the production of single cell oil (SCO) with the yeast *Cryptococcus curvatus* in chapter 5. In contrast to plant oils, microbial lipids can be produced independently from season, climate and location; they do not require arable land and a wide range of carbon sources, e.g. waste streams from food industry or renewable carbon sources, can be used for their production. As well, they often have a fatty acid composition similar to plant oils. By combining the produced SCO with the results from chapter 4, it was possible to produce enzymatically a glycolipid-mixture, completely based on sustainable resources. These glycolipids were identified via TLC, various mass analysis methods and NMR.

Next to lignocellulose, there are many other potential natural substrates for glycolipid production. Alternatives could be honey or agave syrups, which consist of more than 80 % various sugars. They are used as sweeteners, and are ingredients of cosmetics or medical ointments. Furthermore, both have low water content, are often liquid at room temperature and resemble some known sugar-based deep eutectic solvents. For this purpose, important characteristics of the herein used honey and agave syrup were

determined and compared with other available types in chapter 6. Subsequently, an enzymatic transesterification of four fatty acid vinyl esters was accomplished in ordinary honey and agave syrup. Notwithstanding of the high water content for transesterification reactions of the solvent, a successful sugar ester formation was proved by thin-layer chromatography and compared to a sugar ester which was synthesized in a conventional deep eutectic solvent. For a clear verification of the sugar esters, mass determinations by ESI-Q-ToF experiments and a NMR analysis were done. These environmentally friendly produced sugar esters have the potential to be used in cosmetics or pharmaceuticals, or to enhance their effectiveness.

Zusammenfassung

Tenside sind eine vielfältige Gruppe von amphiphilen Molekülen, die die Oberflächenspannung reduzieren und als Waschmittel, Emulgator oder Netzmittel wirken können. Aufgrund dieser Eigenschaften sind sie ein bedeutender Wirtschaftsfaktor und werden im Millionen Tonnen Maßstab produziert.

Tenside sind ein gutes Beispiel für das derzeitige Umdenken in unserer Wirtschaft. Früher wurden Tenside ausschließlich chemisch auf Rohölbasis produziert. Heute jedoch werden dazu häufig erneuerbare Rohstoffe eingesetzt und umweltfreundliche enzymatische Syntheseverfahren zu ihrer Herstellung angewandt oder etabliert.

Diese Arbeit ist eingebettet in das BBW ForWerts Graduiertenprogramm des Landes Baden-Württemberg (Bioökonomie Baden-Württemberg: Exploring Innovative Value Chains). Deren Ziel ist die enzymatische Synthese neuartiger und nachhaltiger Tenside, vor allem von Glykolipiden aus erneuerbaren Substrate in umweltfreundlichen Medien.

Glykolipide sind Tenside mit einem polaren Zuckermolekül als Kopfgruppe und einem hydrophoben Schwanz. Sie zeigen verschiedene nützliche Eigenschaften wie eine geringe Toxizität, sie sind gut biologisch abbaubar und weisen eine hohe Oberflächenaktivität auf, meist in Kombination mit einem niedrigen CMC-Wert. Sie können chemisch oder durch eine umweltfreundliche enzymatische Reaktion hergestellt werden. Dazu wird ein nahezu wasserfreies Lösungsmittel benötigt, um das Reaktionsgleichgewicht in Richtung Acylierung zu verschieben. Als Substrate kommen verschiedene Kohlenhydrate in Frage, die in den benötigten wasserfreien unpolaren organischen Lösungsmitteln jedoch schwer löslich sind. Daher wurde in dieser Arbeit (Kapitel 2) ein umweltfreundliches, tiefes eutektisches Lösungsmittelsystem (DES) für die enzymatische Glykolipidsynthese etabliert. Durch Mischen und Erhitzen des Ammoniumsalzes Cholinchlorid und eines Zuckers im geeigneten Molverhältnis wurden mehrere DES gebildet. Das so gebildete DES wirkt simultan als umweltfreundliches Reaktionsmedium und Substrat für eine lipasekatalysierte Synthese von Glykolipiden. Insgesamt wurden fünf Zucker, drei Zuckeralkohole und ein Anhydrozucker in Kombination mit bis zu neun Fettsäuren verwendet. Die erfolgreiche Bildung von Tensiden wurde durch Dünnschichtchromatographie, Massenspektrometrie und NMR-

Analysen nachgewiesen. Zudem wurde erstmals ein DES aus einem Anhydrozucker hergestellt und darin eine erfolgreiche enzymatische Synthese durchgeführt.

In Kapitel 3 wurden die zeitabhängigen Produktbildung und Reaktionsausbeuten von Arabinoselaurat und –dilaurat bestimmt, welches als Referenzglycolipid dient. Zuerst wurde das am besten geeignete Verhältnis von Cholinchlorid und Arabinose und als zweites der optimale Wassergehalt bzw. die Wasseraktivität für die DES-Reaktionsmedien bestimmt, um die enzymatische Arabinoselauratsynthese zu optimieren. Als dritter Parameter wurde die Synthesedauer untersucht. Um die zeitabhängige Bildung von Arabinoselaurat und -dilaurat zu bestimmen, wurden mehrere Reaktionen durchgeführt und mittels Hochleistungsdünnschichtchromatographie analysiert. Weiterhin wurden die kritische Micellbildungskonzentration sowie das Potenzial zur Reduzierung der Wasseroberflächenspannung mittels Arabinoselaurat bestimmt. Es wurde gezeigt, dass die Zugabe von Wassermengen von bis zu ca. 8 % die Ausbeute von Arabinoselaurat nach 48 h verdoppeln kann (bis zu 49,6 %); Arabinosedilaurat entsteht hingegen nur, wenn kein zusätzliches Wasser zugegeben wird. Bei höheren Wasserkonzentrationen nimmt die Ausbeute durch die Hydrolyse der Produkte leicht ab. Die meisten Produkte wurden innerhalb von 24 Stunden synthetisiert, längere Reaktionszeiten haben nur einen geringen Einfluss auf die Ausbeute. Darüber hinaus hatte das Verhältnis der DES-Komponenten ebenfalls nur einen geringen Einfluss auf die Ausbeute. Arabinoselaurat weist vielversprechende Eigenschaften auf. Es senkt die Oberflächenspannung von Wasser deutlich auf 23 mN/m senken, wobei der CMC-Wert mit 530 mg/L viel höher als bei anderen bekannten Glykolipiden liegt.

Kapitel 4 behandelt die Verwendung von Zucker aus Buchenholz als nachhaltige Quelle für den Zuckeranteil von Glykolipiden. Die entsprechenden Kohlenhydrate wurden durch einen säurekatalysierten Organosolv-Prozess gewonnen, bei dem Buchenholz in eine Faserfraktion umgewandelt wurde, die anschließend enzymatisch hydrolysiert wurde, um Monosaccharide zu erhalten. Nach der Reinigung und Trocknung wurde diese stark Glukose- und Xylose haltige Fraktion verwendet, um damit ein DES herzustellen. Durch die Verwendung in diesem bereits etablierten Reaktionssystem dient der Zucker gleichzeitig als Lösungsmittel und Substrat für die lipasekatalysierte Reaktion. Schließlich wurde die erfolgreiche Synthese von Glykolipiden durch ESI-Q-ToF Massenspektrometrie und multidimensionale NMR-Experimente bestätigt. Darüber hinaus wurde mittels LC-MS/MS eine Ausbeute von 4,8 % bestimmt.

Um Zuckerester vollständig auf Basis von nachhaltigen Rohstoffen herzustellen, ist eine geeignete Quelle für die Fettsäuren notwendig. Um die Verwendung von Pflanzenölen zu vermeiden, wurde in Kapitel 5 die ungereinigte Monosaccharidlösung aus Buchenholz als Kohlenstoffquelle für die Herstellung von „Single Cell Oil“ (SCO) mit der Hefe *Cryptococcus curvatus* verwendet. Im Gegensatz zu Pflanzenölen können mikrobielle Lipide unabhängig von Jahreszeit, Klima und Standort produziert werden; sie benötigen keine Anbaufläche und eine Vielzahl von Kohlenstoffquellen, z.B. Abfallströme aus der Lebensmittelindustrie, können für ihre Herstellung genutzt werden. Außerdem haben sie oft eine

ähnliche Fettsäurezusammensetzung wie Pflanzenöle. Durch die Kombination des so hergestellten SCO mit den Ergebnissen aus Kapitel 4 konnte enzymatisch ein Glykolipid-Gemisch hergestellt werden, das vollständig auf nachhaltigen Ressourcen basiert. Diese Glykolipide wurden über Dünnschichtchromatographie, verschiedene Massenanalysemethoden und NMR identifiziert.

Neben Lignocellulose gibt es viele andere potenzielle natürliche Substrate für die Glykolipidproduktion. Alternativen könnten Honig oder Agavensirup sein, die zu mehr als 80 % aus verschiedenen Zuckern bestehen. Sie werden als Süßstoffe verwendet und sind Bestandteile von Kosmetika oder medizinischen Salben. Darüber hinaus haben beide einen geringen Wassergehalt, sind oft bei Raumtemperatur flüssig und ähneln somit einigen bekannten zuckerbasierten DES. Um ihre Eignung als Glykolipidsubstrat zu testen, wurde in Kapitel 6 wichtige Merkmale des verwendeten Honig- und Agavensirups bestimmt und mit anderen verglichen. Anschließend wurde eine enzymatische Umesterung von vier Fettsäurevinylestern in normalem Honig und Agavensirup durchgeführt. Ungeachtet des hohen Wassergehalts des Lösungsmittels wurde die erfolgreiche Glykolipidbildung durch Dünnschichtchromatographie nachgewiesen und mit einem Zuckerester verglichen, die in einem herkömmlichen DES synthetisiert wurde. Für einen eindeutigen Nachweis der synthetisierten Glykolipide wurden deren Massen bestimmt und eine NMR-Analyse durchgeführt. Diese umweltfreundlich hergestellten Zuckerester haben das Potenzial, in Kosmetika oder Arzneimitteln eingesetzt zu werden und ihre Wirksamkeit zu erhöhen.

Table of content

1 Theoretical background & research proposal

Parts of this chapter are based on the author's contribution to the book chapter:

Enzymatic Synthesis of Glycolipid Surfactants

In *"Lipid Modification by Enzymes and Engineered Microbes"*, 2018

Sascha Siebenhaller, Jens Grüninger and Christoph Syldatk

Academic Press and AOCS Press

Published Date: 20th March 2018

ISBN: 9780128131671

Surfactants are produced in a million of tons' scale and play an important role in many industrial applications or consumer products (Verband Tegewa e.V., 2014). Until now, most surfactants are synthesized on a chemical route using toxic solvents and energy-intensive processes making the production of sustainable surfactants to an important part of the realignment towards a bio-based economy.

In a bio-based economy renewable resources are used to produce sustainably important platform chemicals, intermediates and value-added products, the usage of dwindling fossil resources like coal, gas and above all crude oil should be avoided. To transfer our economy into a bio-based economy, conventional refineries have to be replaced, or upgraded, stepwise into biorefineries, in which new technologies and approaches of biotechnology and chemistry are combined. Nevertheless, when selecting renewable resources, attention must be paid to the "food or full" discussion. Lignocellulose for example, has the potential to be a suitable raw material for many products, but it has to be pretreated prior use to make sugars accessible for biotechnological and chemical applications (FitzPatrick et al., 2010; Guerriero et al., 2016; Jönsson et al., 2013; Sindhu et al., 2016; Wyman, 1999).

Special attention has to be paid to sustainable reaction media in green biotechnological and chemical processes, which may even be produced from renewable raw materials. Deep eutectic solvents, which are generally considered as green solvents, could be promising candidates to replace hazardous and environmental harmful organic solvents (Cvjetko Bubalo et al., 2015; Dai et al., 2013; Paiva et al., 2014).

1.1 Surfactants

Surfactants are amphiphilic molecules, meaning they contain both a hydrophilic group (head) and a hydrophobic group (tail). Head and tail can consist of one single group or of a complex and branched structure (Figure 1.1). Mostly, the unpolar tail consists of saturated or (poly-) unsaturated alkyl side chains, whereas the chemical structure of the polar head can be much more variable and categorizes surfactants into non-ionic, cationic, anionic or amphoteric subclasses (Table 1.1; Georgiou et al., 1992).

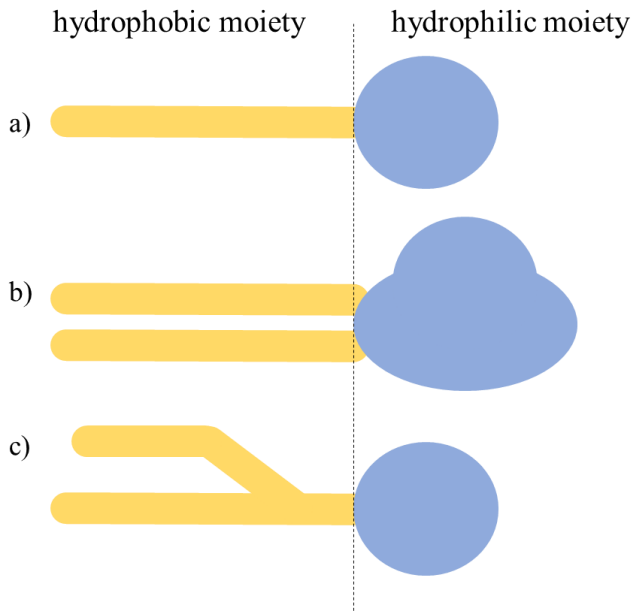

Figure 1.1: Exemplarily schematic structures of surfactants. a) Simple surfactant, consisting of a single tail and head group. Complex surfactants with b) multiple head groups and two tails or c) a branched tail.

Table 1.1: Surfactant classification according to the charge.

Surfactant type	Property	Functional group	Example
Anionic	Negative charge at the hydrophilic head	solfonates (-SO_3^-) carboxylates (-COO-)	Sodium dodecyl sulfate (SDS)
Cationic	Positive charge at the hydrophilic head	quaternary ammonia groups ($^+$N-R_4)	Didecyldimethylammonium chloride
Nonionic	No charge	alcohols (-OH) ether (-O-)	Alkyl ethylene glycol
Amphoteric	Positive and negative charge at the hydrophilic head	combination of both, anionic and cationic groups	Phosphatidylcholine

Due to their structure, surfactants have surface active properties between two liquids, a gas and a liquid, or a liquid and a solid. They will adsorb at interfaces and can reduce the surface tension of aqueous solutions with increasing concentration. If the surface is fully covered by surfactant molecules, surface tension remains stable and thermodynamic favorable self-aggregation leads to the formation of micelles in which the hydrophilic groups point towards the aqueous phase and the hydrophobic tail towards the interior of the micelle. At this point, the so called critical micelle concentration (CMC) of the amphiphilic compound is reached; a further increase of the surfactant's concentration yields in more micelles in the solution and will not further affect the surface tension (Figure 1.2). For most applications, like in cleaning products, low CMC values and a strong reduction of the surface tension are preferred due to a better removal of impurities. Surfactants can be grouped be their specific CMC value which is depending on the polar head and the length of the hydrophobic tail (Tadros, 2006). Micelles can occur

in various shapes (e.g. cylindrical or spherical), cohered by Van-der-Waals forces, hydrogen bridges, hydrophobic- and electrostatic interactions, influenced by the surfactants head and tail (Lin, 1996).

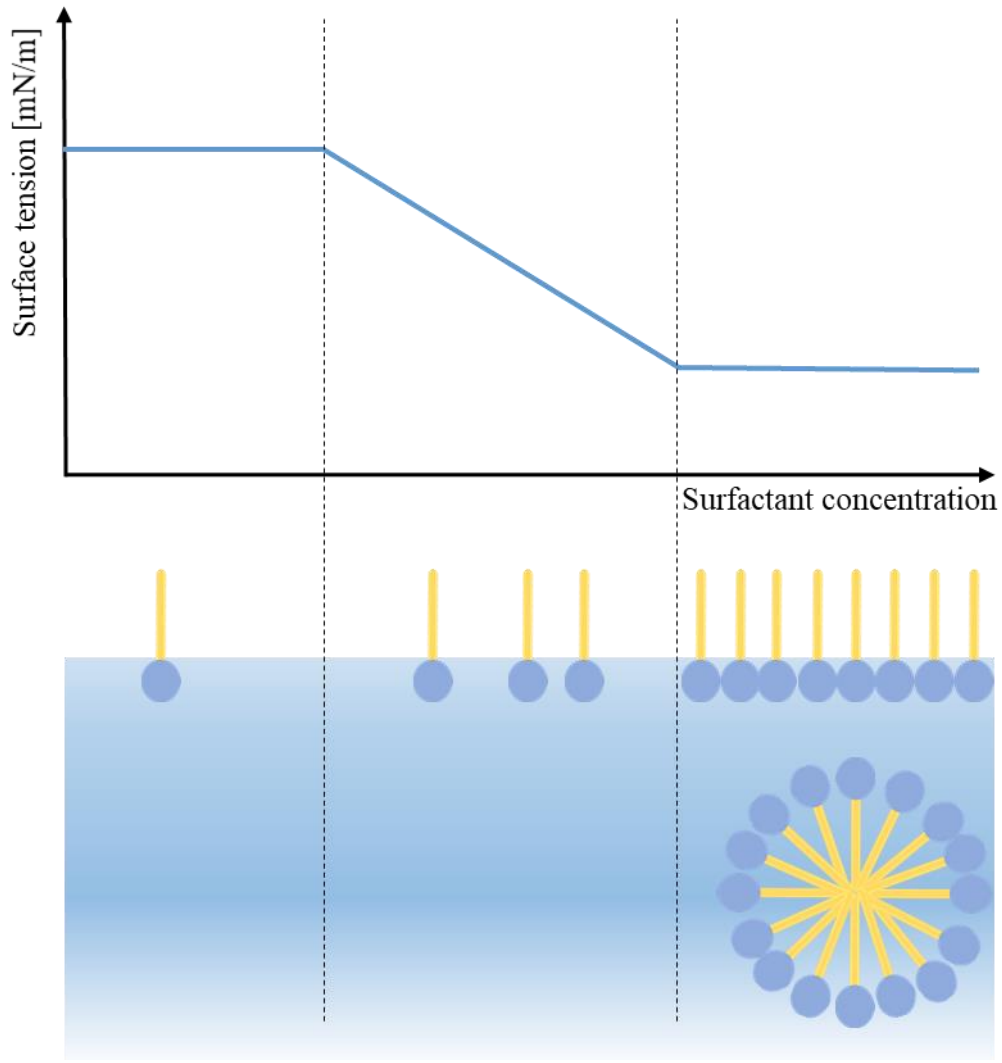

Figure 1.2: Correlation between surfactant concentration, surface tension und formation of micelles on an air-water interface.

Surfactants have the ability to be used as foaming- and wetting agents, detergents, emulsifiers and dispersants (Lin, 1996; Rosen, 2004). Therefore, they are used in many products such as in cleaning and personal care products, cosmetics, food, pharmaceuticals, mining and oil production (Figure 1.3). These applications make surfactants to a very important industrial class of substances, of which over 13 million tons were produced in 2008 with annual global sales of over 23 billion dollars (Acmite Market Intelligence, 2008; Reznik et al., 2010). Further, it is expected that the production of surfactants and their sales will increase to over 40 billion dollars by 2022.

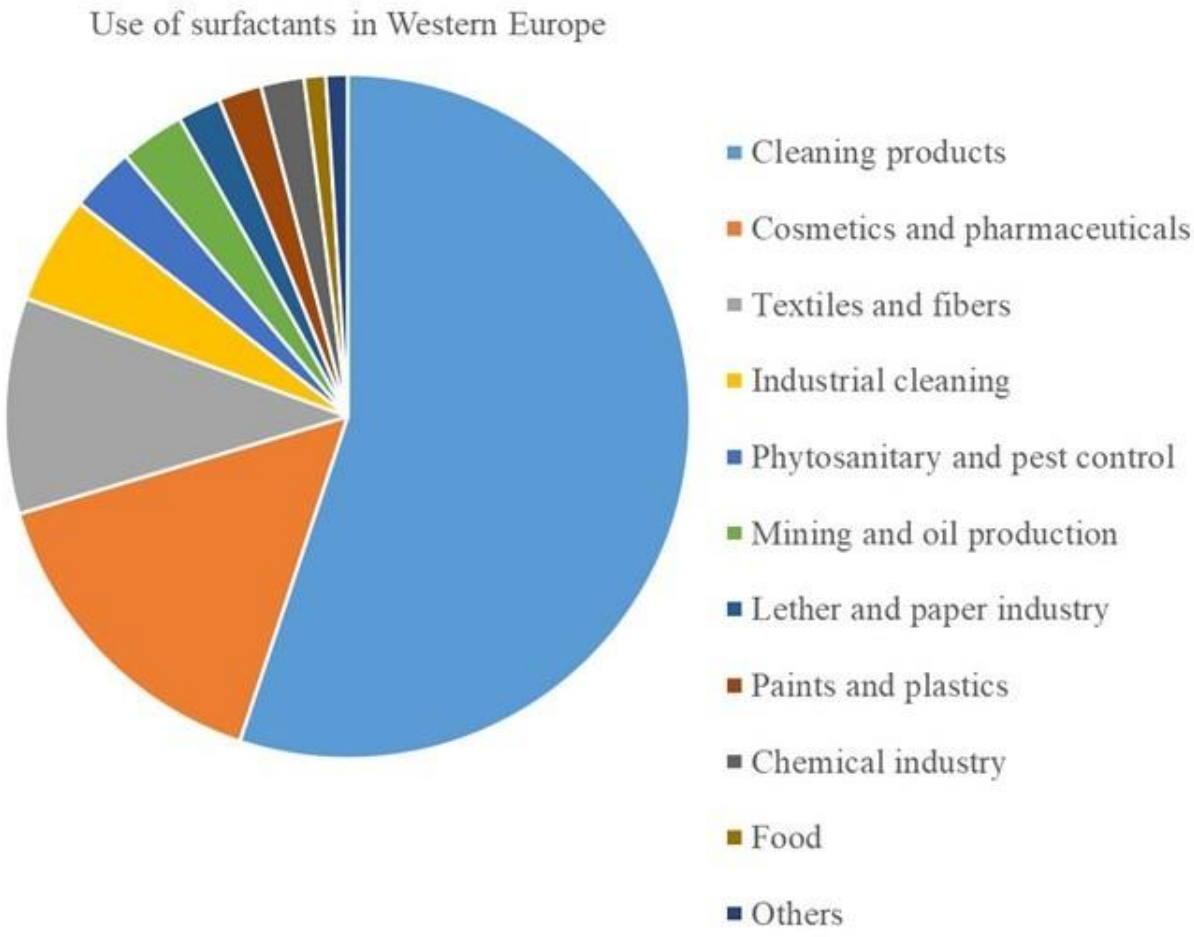

Figure 1.3: In Western Europe, a total of around 2.5 million tons of surfactants are consumed each year in the specified areas (share of total consumption in %, shares constant over the past few years) (Verband Tegewa e.V., 2014)**.**

Even today, in a society that places great emphasis on environmental protection and sustainability, most surfactants are produced chemically and on the basis of fossil raw materials. Todays most produced surfactants are linear alkylbenzene sulfonates (LAS), which are biodegradable in the present of oxygen, but are completely produced based on petrochemicals (Saouter et al., 2006). Further, their production process involves the alkylation of benzene, sulfonation of the resulting alkylbenzene and a neutralization under partially high temperatures and pressure (Kosswig, 2012). In contrast to LAS, currently around 50 % of all surfactants have at least one group, which originates from a renewable resource (Verband Tegewa e.V., 2014). Mostly, the hydrophobic tail is derived from palm or coconut oil (Patel, 2003), and in a position paper from 2010 it is stated, that over one third of vegetable fats and oils processed by industry are used for the production of surfactants ("Rohstoffbasis im Wandel," 2010). Vice versa, one obstacle of the use of these vegetable oils is, that forests often have to be cleared for their cultivation which leads to massive environmental damage (Carlson et al., 2012).

Like LAS, other surfactants synthesized via chemical routes offer high yields and a cheap production, but their production needs harmful chemicals and produces high amounts of toxic waste. Further, during chemical synthesis side products with vastly different and possible hazardous properties can be formed (Banat et al., 2000).

1.1.1 Biosurfactants

Biosurfactants are an alternative to chemically produced surfactants. They are a structurally diverse group of naturally occurring surface-active molecules, produced mainly, but not exclusively by bacteria and fungi (Marchant and Banat, 2012a). In the last decades, biosurfactants were gaining considerably more attraction, especially in the food, pharma and oil industry (Banat et al., 2000; Desai and Banat, 1997). Biosurfactants are considered to have an environmental friendly nature, they are easily biodegradable, non-toxic and have good physico-chemical and biological properties (Nitschke and Costa, 2007; Van Bogaert et al., 2007). Most biosurfactants can be produced either by microbial cultivation or they can be re-produced synthetically (Müller et al., 2012). They are classified by their structure and surfactant properties (Lang and Trowitzsch-Kienast, 2002) or into high- and low molecular mass molecules (Rosenberg and Ron, 1999). When producing biosurfactants by cultivation, it is possible to use agro-industrial by-products or wastes, like glycerol from the biodiesel production or distillery and dairy wastes (Makkar and Cameotra, 2002; Nitschke and Costa, 2007). Until now, a few processes are commercialized to produce rhamnolipids or sophorolipids, but the downstream processing is very costly. A further drawback of microbial fermentations is, that they are leading to product mixtures and different compositions, depending on the strain, substrates and fermentation conditions (Siebenhaller et al., 2018). Moreover, the microbial produced structure diversity of biosurfactants is limited and an extension is lavishly. The usage of different enzymes allows the modification of microbial glycolipid surfactants in order to obtain novel structures with new properties, which might have the potential to alter and improve the physicochemical characteristics of known glycolipids to fit better to a desired application (Marchant and Banat, 2012b; Müller et al., 2012).

1.1.2 Glycolipids

Glycolipids are sugar esters and the main topic of this thesis. They belong to the class of low molecular weight biosurfactants. They are characterized by having a mono-, di- or oligosaccharide as polar head while having a hydrophobic tail of one or more alkyl moieties, which can vary in chain length. Some glycolipids, like the mentioned rhamnolipids from *Pseudomonas aeruginosa* or sophorolipids produced by *Candida bombicula*, are well characterized, highly surface- and sometimes bioactive and exhibiting very low CMC values. Glycolipids have beneficial characteristics, like being biodegradable, non-toxic, odor- and tasteless and non-irritant, making them very interesting for applications in the cosmetic and food industry for example as emulsifiers (Coulon et al., 1996; Recke et al., 2013; Tarahomjoo and Alemzadeh, 2003). Additionally, antimicrobial (Altenbach et al., 2010), antiviral (Harada et al., 2007), antifungal (Teichmann et al., 2007) and anticancer (Recke et al., 2013) properties of glycolipids are reported in the literature, making them to an interesting field of research.

In contrast to chemical synthesis and microbial fermentation processes with subsequent enzymatic modification, the direct enzymatic coupling reaction between sugars and fatty acids or fatty alcohol derivatives allows the synthesis of new and unexplored sugar esters. For this purpose, hydrolytic enzymes can be used as biocatalysts, which have the capability, under conditions of reduced water activity or at very high substrate concentrations, to reverse their hydrolytic activity, synthesizing ester-, peptide- or glycosidic bonds (Zaks and Klibanov, 1986). As an example, the enzymatic acylation of sugars bears the possibility to obtain a high diversity of glycolipid surfactants, since theoretically the combination of every sugar with every fatty acid derivative should be possible. With the knowledge and understanding of these enzymatic synthesis processes, it should be possible to take an influence on the final product and its surfactant properties by selecting suitable building blocks.

1.2 Enzymes

Enzymes are proteins, which accelerate chemical reactions as a biological catalyst, either inside or outside a living cell. Each enzyme consists of a specific amino acid sequence, arranged to a complex three-dimensional structure. They can bind certain substrates effectively, stabilize their transition states and convert them selectively and specifically to a distinct product, without any need for a high energy input (Faber, 2011). Thus enzymes enable the use of mild reaction conditions (temperature, pH value, pressure) in almost all solvents, which clearly distinguishes an enzymatic from a chemical process (Faber, 2011).

In general, enzymes are classified in six main classes (oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases), based on their catalyzed reaction of which more than 5,000 different reaction types are known (Schomburg et al., 2013), making them to a universal tool in the chemical-, biological-, food- and pharma industry.

One very important and often used class of enzymes are hydrolases (E.C. 3.x.x.x). They use water as co-substrate to act on chemical bonds; the most common reaction is the cleavage of a chemical bond by the addition of water (Figure 1.4).

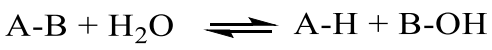

Figure 1.4: Reaction scheme of a hydrolysis reaction. The chemical bond A-B gets cleaved by the addition of water. Vice versa, the reversed reaction is possible, depending on the reaction equilibrium.

Unlike many other enzymes, hydrolases lack of sensitive cofactors which makes them the most commonly used enzymes in biotransformation (Bornscheuer UT, Kazlauskas RJ (2006) Hydrolases in Organic Synthesis, 2nd ed. Wiley-VCH, Weinheim, 2006). Further, some hydrolases are able to reverse their natural hydrolytic activity in a nonaqueous media, what allows them to synthesize chemical bonds, making these enzymes valuable for synthesizing glycolipids.

Hydrolases are further classified into several subclasses like esterases (E.C. 3.1.x.x), glycosidases (E.C. 3.2.x.x) or peptidases (E.C. 3.4.x.x), according to the bonds they act on.

1.2.1 Lipases

Lipases (E.C. 3.1.1.3) were the first hydrolases described with synthetic activity in water-free organic solvents (Zaks and Klibanov, 1984). They preferentially cleave triglycerides by hydrolysis, a common reaction employed in nearly all organisms. Most lipases show an increased activity due to a change in conformation, when they are acting at an interface: normally a *lid* covers the enzymes active center with its catalytic triad, contact to an unpolar solvent changes the enzymes conformation and open it (Aloulou et al., 2006; Meier et al., 2007).

Lipases are widely used in industrial processes like the production of biodiesel, flavor and fragrance esters (acetic or butyric acids or geraniol) or wax esters, the synthesis or modification of triacylglycerols or the racemic resolution of chiral esters and amines (Adlercreutz, 2013; Bajaj et al., 2010; Larios et al., 2004; Öhrner et al., 1996). A further application of lipases is the modification of carbohydrates, leading to surface active glycolipids (Gumel et al., 2011; Pöhnlein et al., 2015a).

One of the most common used lipase is the *Candida antarctica* lipase B (CalB). CalB has a molecular weight of 33 kDa and is composed of 317 amino acids (Uppenberg et al., 1994) and lacks interfacial activation. This lipase is characterized by a large number of beneficial properties. The optimal pH is 7, but it is stable in aqueous media in a wide range between pH 3.5-9.5. CalB is thermostable up to 60 °C, at lower pH the denaturation temperature is higher (Anderson et al., 1998). CalB is often immobilized on different macro-porous carriers like acrylic resins, having a stabilizing effect and allowing an easy enzyme recovery. Immobilized CalB (iCalB or the trade name “Novozyme 435”) has a higher thermostability without any significant loss in activity after several thousand hours in a suitable solvent (Heldt-Hansen et al., 1989). Like most hydrolases, CalB is able to reverse its activity in media with a low water amount (Vescovi et al., 2017). Further, this lipase does not need any co-factors, making it an appropriate enzyme in industry. CalB has only a low affinity to tall molecules like triglycerides, but accepts a wide range of smaller esters, amides and thiols (Anderson et al., 1998). Whether the lipase favors short-, mid- or long-chain fatty acid, can’t be answered in general. Depending on the desired reaction and solvent, CalB sometimes favors short- or long-chain fatty acids (Larios et al., 2004; Selmi et al., 1998; Soultani et al., 2001).

To avoid confusion it is mentioned here that *Candida antarctica* was renamed as *Moesziomyces antarcticus* in 2015 (Wang et al., 2015), but *Candida antarctica* is still a valid synonym (“mycobank.org,” 2018).

In general, enzymes can help with their specificity, selectivity and their required mild reaction conditions, to replace chemical processes with environmentally friendly, sustainable and less energy-intensive enzymatic processes. Therefore, one possible industrial application for lipases is the synthesis of sustainable glycolipids made from renewable resources, which is explained here in detail.

1.3 Enzymatic glycolipid synthesis

Since Klibanov and co-workers described in the 1980s enzymatic activity in water-free non-polar organic solvents (Zaks and Klibanov, 1985, 1984), various hydrolases have been successfully used in different media and with various substrates to form a linkage between a sugar and a carbohydrate chain (Figure 1.5; Degn et al., 1999; Degn and Zimmermann, 2001; Fukuoka et al., 2011; Hyun et al., 2008; Šabeder et al., 2006). Using already described lipases for glycolipid synthesis, during esterification or transesterification an ester bond will be formed.

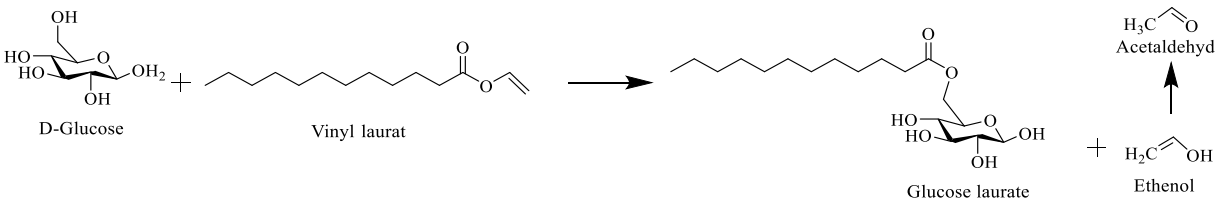

Figure 1.5: Lipase-catalyzed esterification between glucose and vinyl laurate leads to the formation of glucose laurate and ethenol. The vinyl alcohol tautomerizes to acetaldehyde and evaporates, which prevents the back reaction. It might be possible that more or other C-atoms are acylated, too. The same scheme applies to other sugars fatty acid vinyl esters.

This thesis focus on the synthesis of sustainable glycolipids with iCalB in non-conventional media. Therefore, the next chapters describe several possible substrates, reaction media and conditions which can influence the successful enzymatic synthesis.

1.3.1 Substrates

Any combination of a carbohydrate and a fatty acid should be possible for glycolipid synthesis, which makes selection a crucial step. Choosing the right substrate is of elementary importance as it also affects the product properties like the hydrophilic-lipophilic balance (HLB), which allows a classification of the surfactants function. Due to this large variety of substrates, one should think about incorporating cheap or renewable raw materials into the selection. When selecting renewable raw materials as substrate, the fuel or food discussion should take into account. It should further be noted that even renewables are not suitable for the environment if forests have to been cleared for their cultivation as it is often the case, for example, in palm oil production.

a) Sugars

As the name implies, the polar head of a glycolipid is a sugar. Sugars are divided in three different classes depending on their degree of polymerization: sugars (1-2), oligosaccharides (3-9) and polysaccharides (>9) (FAO, 1997). Various sugars from all classes have been used in countless experiments to synthesize the most varied sugar esters (Abdulmalek et al., 2016; Horchani et al., 2010; Hsieh et al., 2015; Plou et al., 2002; Sebatini et al., 2016).

Sugars are naturally produced by plants, often as a storage compound. The two most important sugar producing plants are sugarcane, especially in subtropical countries like Brazil, India and Thailand, and sugar beet, which is mainly produced in Russia, France, USA and Germany (Table 1.2).

Table 1.2: Worldwide production of sugarcane and sugar beet in 2016 ("FAO Produktionsstatisken (FAOSTAT)," 2016).

Sugarcane		**Sugar beet**	
Country	**value [t]**	**Country**	**value [t]**
Brazil	768,678,382	Russia	51,366,830
India	348,448,000	France	33,794,906
China	122,663,940	USA	33,457,880
Thailand	87,468,496	Germany	25,497,200
total	1,890,661,752	total	277,230,789

A detour in the production of sugar is the cultivation of starchy plants such as corn or potatoes. Starch is a glucose polymer, which can cleaved into glucose molecules. This glucose can be processed by a glucose isomerase to high-fructose syrup, a very important sweetener (Hobbs, 2009).

Although these sugars are produced from renewable raw materials, they are usually not sustainable. Huge areas are used for their cultivation (267.743 km^2 are required for sugarcane cultivation, approx. 75 % of the area of Germany), forests are cleared, water is often wasted or pesticides are used. Further, a high amount of so produced glucose is used to produce ethanol as fuel or fuel additive, continuing the food or fuel discussion.

A further renewable resource for sugar production is lignocellulose. As the major structural component of woody and non-woody plants, lignocellulose is the most abundant organic material in the world (Howard et al., 2003; Zhao et al., 2009). It is estimated that the worldwide production of lignocellulose ranges from 120-140 billion tons per year, which is over half of the world's biomass (Pauly and Keegstra, 2008). Lignocellulose is widely used for burning and heating but ultimately it is too valuable for that due to its high potential for material use. Lignocellulose has three major components: lignin, cellulose and hemicelluloses, with carbohydrates accounting for up to 75% of the total amount (Jørgensen et al., 2007). In contrast to renewable resources of the first generation, lignocellulose often occurs

as garden waste or as residue in various branches of industry, so these sugars do not compete with the food, feed or fuel industry and do not have to be cultivated separately like corn or sugarcane, making it more sustainable. In order to utilize these huge quantities of sugars, a pretreatment is necessary because of the recalcitrance of the lignocellulose and to make the carbohydrates available for succeeding hydrolysis (Rubin et al., 2007). To achieve this, many processes were invented in the last decades to make the sugar accessible. One of them applied within the frame of this work is a chemical-physical process which uses an aqueous-organic solvent system under high pressure and temperature to separate the lignin from the carbohydrates (Figure 1.6; Kumar et al., 2009; Laure et al., 2014; Pan et al., 2008). The obtained cellulose fiber fraction can be subsequently saccharified enzymatically yielding a glucose-rich hydrolysate, which is highly suitable as a microbial carbon source, as a building block or as a pre-cursor for other (fine-)chemicals (Dörsam et al., 2017; Laure et al., 2014; Nigam, 2001).

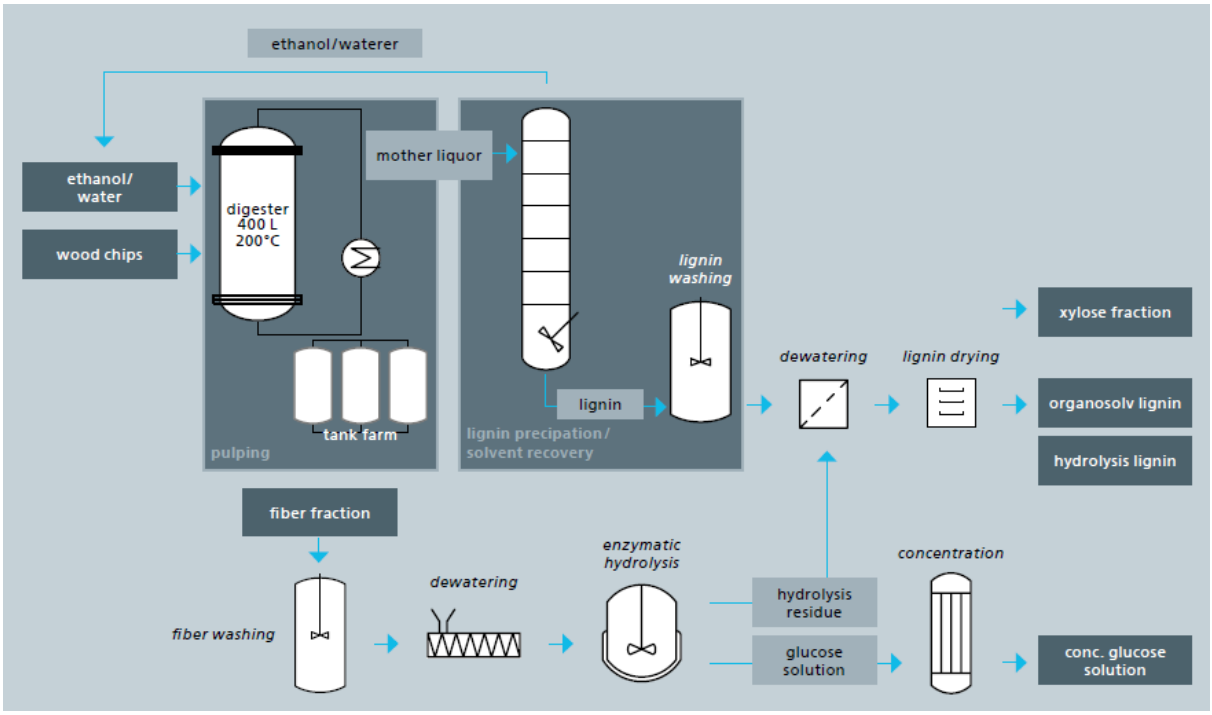

Figure 1.6: Simplified flow chart of the lignocellulose biorefinery pilot plant in Leuna, Germany. First, lignocellulose is pulped, whereby lignin and hemicellulose are dissolved in the ethanol-water mixture (mother liquor). Lignin is precipitated by adding water or distillation of the ethanol with subsequent filtration, washing and drying. The hemicellulose sugars remain as an aqueous solution, which can be further processed. The solid fiber fraction is washed and dewatered. Enzymes can be added for saccharification of the celluloses glucose. After a final filtration and concentration step, a glucose-containing syrup is formed (Leschinsky, 2018)**.**

However, it is not as simple as it seems to use sugars as substrates for all chemical or enzymatic processes. Without exception, all sugars have a low solubility in non-polar organic solvents which is challenging for a large scale industrial production. To overcome this obstacle and to improve the yields, several parameters can be modified to increase the solubility of sugars. The easiest way is the addition of solvents to the reaction, which will improve the availability of sugars. Dimethyl sulfoxide (DMSO) and Dimethylformamide (DMF) can achieve this, but also can inhibit the enzyme performance. Another

way to enhance the sugar solubility is to employ hydrophobic methylated or acetylated sugars. It is also possible to dissolve the sugar in water or *n*-butanol before adding it to the reaction mixture, since the solubility of glucose in water is 470 g/l. Crout et al. showed that an enzyme-catalyzed synthesis between glucose and 2-hydroxybenzyl alcohol is possible (Vic et al., 1997).

b) Fatty acids

For an enzymatic synthesis of a glycolipid the selection of the fatty acid should also be well considered.

First, the chain length will influence the characteristics of the sugar ester like the HLB, CMC and foam properties (Kölbel and Kühn, 1959). Second, the fatty acid will have an impact on reaction parameters and the yield. A study in 2002 investigated the effect of fatty acid length on initial rates and yields when using iCalB. They found out, that shorter fatty acids increased the yield and the enzymes initial rate (Pedersen et al., 2002). A general statement cannot be made, however, since the respective result is influenced by too many parameters like enzyme, solvent, temperature, etc. Third, the usage of fatty acid esters like fatty acid methyl-, ethyl-, or vinyl esters can support the reaction and shift the reaction equilibrium favorably. The kind of reaction will switch from an esterification to a transesterification, which is easier to catalyze by most enzymes. Further, water will not be a side product, which could prevent a subsequent hydrolysis of formed sugar esters. When using a vinyl ester like in this study, the emerging vinyl alcohol (ethenol) will tautomerize to the more stable acetaldehyde. Acetaldehyde evaporates at 20 °C and can thereby help to push the reaction equilibrium towards glycolipid formation. A drawback of the usage of fatty acid esters is, that side products like methanol or acetaldehyde can lower the reaction rate by inhibiting or denaturing the enzyme (Bélafi-Bakó et al., 2002; Weber et al., 1995).

Here it has to be mentioned, that the effect of water on enzymatic reactions has not been finally clarified.

A large percentage of fatty acids for glycolipid production is based on fossil substances. Alternatives for this are natural fatty acids derived from vegetable oils. It should be considered, whether the plants for this purpose were cultivated sustainably or not. Several studies investigate culture conditions and potential substrates to produce oils from microorganisms (Ratledge, 2004). As benefit, these oils can be produced season-independently and from waste materials or renewable carbon sources and can be potentially used for the production of sugar esters (Economou et al., 2011; Fakas et al., 2009).

1.3.2 Reaction media

The selection of the reaction medium is extremely important. It will have a high impact on the stability and activity of the enzyme and a not negligible influence on the solubility of the substrates. Furthermore, the water-content, boiling point, possible downstream processes and the environmental sustainability of the solvent have to be considered as well.

a) Organic Solvents

Organic solvents can be divided into two groups, depending on their behavior in a mixture of a polar (water) and a nonpolar phase (e.g. octanol). The resulting factor, the so-called logP value, distinguishes between nonpolar solvents with a positive logP and polar solvents with a negative logP. Solvents with low logP interfere with the enzymes hydration state and remove water from it, leading to changes in the enzyme structure and its inactivation (Gorman and Dordick, 1992).Vice versa, unpolar solvents with a high logP value will not interfere with the enzymes hydrate shell, so that the enzyme stability can be even higher in comparison to water (Gupta, 1992). It is widely accepted that hydrolases like lipases and esterases show a good activity in solvents with a high logP value (Laane et al., 1987). A drawback for the enzymatic synthesis of sugar esters in organic solvents is the poor solubility of sugars in them. Further solvent parameters, such as the dielectric constant, describing the solvents relative permittivity, the electron acceptance index as well as the Hildebrand parameter and the dipole moment can help to predict the interactions of the solvent with the enzyme and substrate (Ebert et al., 1996; MacManus and Vulfson, 1997; Stergiou et al., 2013).

For the enzymatic synthesis of glycolipid surfactants, first solvents like pyridine, dimethyl formamide or dimethylpyrrolidone were used due to the good solubility of carbohydrates (Klibanov, 1989; Therisod and Klibanov, 1986), despite of their toxicity. In the following years, several solvents with logP ranging from very hydrophobic like hexane to rather hydrophilic solvents like acetone have been tested to find a suitable solvent (Yan et al., 1999).

The most suitable organic solvents seem to be tertiary alcohols like tert-butanol, 2-methyl-2-butanol (2m2b) and related ones. They provide a high enzyme stability and activity and occur in many natural formulations, allowing their use in the food industry. Another benefit of tertiary alcohols is their sterically constraints, which prevents them not being a substrate for the used enzyme. Testing four immobilized lipases in three different solvents for the production of lactose-mono-laurate, 2m2b showed the highest yields (Walsh et al., 2009).

b) Deep eutectic solvents

Deep eutectic solvents (DES) are a novel solvent system for enzymatic reactions, first described by Abbott et al. (2002). DES consists of a hydrogen bond acceptor (HBA), e.g. an ammonium- or phosphonium-salt and one or a mixture of hydrogen-bond donors (HBD) like alcohols, amines, amides, sugars or a carboxylic acid (Figure 1.7; Abbott et al., 2004). By mixing and heating these components in a certain molar ratio, a liquid is formed, which stays liquid at lower temperatures. The melting point decreases due to intermolecular hydrogen

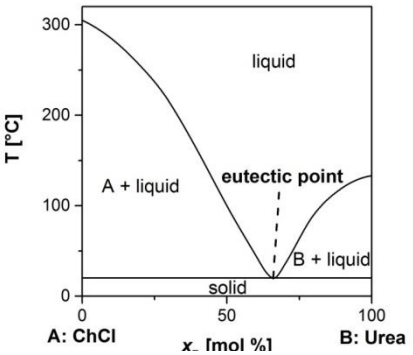

Figure 1.7: Schematic phase diagram of the eutectic mixture of CC:Urea 1:2 (mol:mol). (Smith et al. 2014).

bonds between the HBD and the HBA (Durand et al., 2012; Pena-Pereira and Namieśnik, 2014; Tang and Row, 2013).

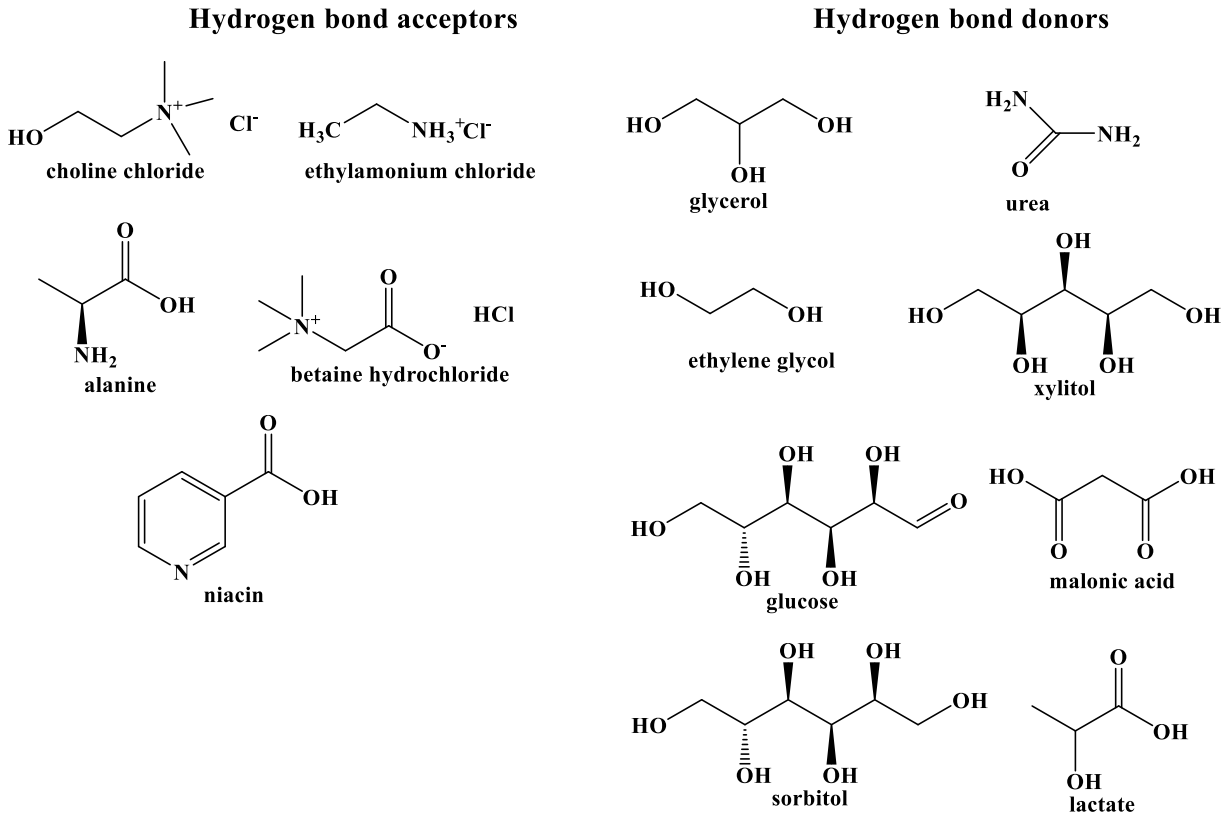

Figure 1.8: Frequently used HBA and HBD for the production of DES.

In literature DES are often counted as an ionic liquid (ILs = liquid salts whose ions hinder the formation of a stable crystal lattice by charge delocalization and steric effects). This is in most cases not correct and can be confusing, but in a few cases it is correct because some DES and IL share the same principle of creation (Durand et al., 2016). In contrast to organic solvents, DES and IL have a low vapour pressure, are not flammable and are often stable at high temperatures (Dominguez de Maria and Maugeri, 2011). Nevertheless, ILs offer drawbacks like an often complicated formation, the components should be very pure and therefore are often expensive, they show eco- and cytotoxicity and a low biodegradability (Cvjetko Bubalo et al., 2015; Park and Kazlauskas, 2001; Thuy Pham et al., 2010). However, DES can be produced e.g. by inexpensive quaternary ammonium salts like choline chloride (also called vitamin B4, which is produced easily and in million tons per year (Zhao and Baker, 2012), by renewable resources like sugars or alcohols or by process wastes like glycerol. Therefore, DES are generally considered as a green, non-toxic and biodegradable solvent class, but a final general assessment has not yet been completed (Kim et al., 2016). In 2015 it was shown that, depending on the HBA and HBD, DES can have a low to moderate cytotoxicity, showing antibacterial and toxic effects on selected living organisms and are not readily biodegradable (Radošević et al., 2015; Wen et al., 2015). Further Hayyan et al. reported that the cytotoxicity of a DES is higher than of their individual components, indicating, that

the use of the terms non-toxic and biodegradable should be used with caution (M. Hayyan et al., 2013), but the toxicity and biodegradability of most IL and organic solvents is much worse.

Until now, several publications show the suitability of DES as a potent class of solvent for the enzymatic synthesis of various chemicals or biodiesel (E. Durand et al., 2013; Liu et al., 2015; Niawanti et al., 2017). In 2015 the possibility of synthesizing the glycolipid glucose hexanoate in a DES consisting of choline chloride and urea by using vinyl hexanoate as fatty acid vinylester and Novozyme 435 as catalyst was demonstrated (Pöhnlein et al., 2015b). Furthermore, the authors revealed that it is possible to use a DES, based on choline chloride and glucose, which acts as solvent and substrate simultaneously. One challenge of this sugar containing system is its high viscosity, making a good intermixing difficult (A. Hayyan et al., 2013). To overcome the high viscosity of DES, it is possible to mix a DES with 2m2b (Zhao et al., 2016) or to add small amounts of water (Erwann Durand et al., 2013).

1.3.3 Reaction conditions

For an efficient enzymatic synthesis, process parameters like temperature, water content and the ratio of substrates are rudimental.

Lipases are stable in organic solvents and they can also withstand slightly higher temperatures, but their thermostability can be enhanced drastically by immobilization. Most commercially available immobilized lipases are stable between 20 °C and 60 °C, some others like Novozyme 435 can be used in special cases up to 80 °C without a significant loss in activity (Poojari and Clarson, 2013). Thus, a wide range of temperatures should be tested to find optimal reaction conditions for glycolipid synthesis. Higher temperatures might boost the reaction rate of the enzyme and support the solubility of the carbohydrates, but at the same time changes in the reaction equilibrium and enzyme selectivity could be influenced. In addition, the enzyme can denature at too high temperatures, the solvent can evaporate or burn and the product can be deteriorated.

The molar ratio between the sugars and the fatty acids is another parameter which influences the enzymatic synthesis of glycolipids. Using fatty acids in excess may have an inhibiting effect on the enzyme (Chamouleau et al., 2001). Additionally, the proportion of formed mono-, di- and poly-acylated sugars also depends on the ratio of the substrates used (Cauglia and Canepa, 2008). It has not yet been fully clarified yet what is the most promising ratio for the highest yields. The best substrate ratio can also depends on factors like the chosen fatty acid chain length (Sakaki et al., 2006). In the synthesis of novel glycolipids, several variations of all parameters must therefore be considered in order to optimize the synthesis reaction.

So called anhydrous organic solvents like organic solvents, DES or ILs still contain small amounts of bound and unbound water despite being called anhydrous. The water is crucial to maintain the enzymes

3D structure and flexibility (Amos et al., 1998). Thus, the water content in these solvents, respectively the water activity (a_w), which is a thermodynamical parameter and is defined as the correlation of the vapor pressure in the system and the saturation vapor pressure of pure water at a defined temperature, are essential for the activity of enzymes and their hydrate shell (Monsan and Combes, 1984; Zaks and Klibanov, 1988). Consequently, only suitable combinations of enzymes and solvents with distinct water activities enable enzymatic syntheses in organic solvents.

Chamouleau et al. reported, that the synthesis of fructose palmitate with Novozyme 435 in 2m2b has the highest reaction rate at a_w values < 0.2 (Chamouleau, 2001). Further, reaction yields as well as enzyme stability and selectivity are also controlled by the water activity.

Water content and activity of a reaction mixture can be regulated before and during the reaction (Bell et al., 1995). Molecular sieves with a pore size of 3-4 Å can remove water from the reaction media by adsorption, thus keeping the water activity low throughout the reaction (Khan et al., 2015; Plou et al., 2002). Another strategy could be to work in vacuum or under reduced pressure as well as the removal of unwanted side product water by azeotropic distillation (Sarney et al., 1997; Yan et al., 1999).

1.4 Research proposal

The global demand for surfactants is rising, while at the same time the economy has to be changed into a bio-economy. Therefore, there is a particular demand for environmentally friendly surfactants that can be produced sustainably. Glycolipids could assume this role, because, as shown in the theoretical background, they generally have many beneficial properties and can be tailored enzymatically to address specific applications due to their structural diversity.

The enzymatic synthesis of glycolipids, unlike the chemical production of surfactants, can become part of a green and sustainable bioeconomy if some points are fulfilled:

- Selection of suitable substrates
- Usage of an environmentally friendly reaction media
- Abandonment of chemical synthesis pathways and the use of enzymes

These three points were considered in the preparation of this doctoral thesis, which resulted in the following research packages:

- Establishing a sustainable deep eutectic solvent system for the production of glycolipid surfactants
- Determination of the DES properties and optimization of critical reaction parameters
- Enzymatic synthesis of surfactants like glycolipids and sugar alcohol esters in the DES reaction system with commercially available substrates
- Usage of renewable sugars from beech wood as substrate
- Production of single cell oil with yeasts based on beech wood cellulose fiber hydrolysate
- Development of an integrated process for the enzymatic production of glycolipids completely based on lignocellulosic substrates
- Usage of natural sugary liquids as simultaneous media and substrate
- Mass and structure elucidation of synthesized surfactants
- Ascertain the surface properties of surfactants using arabinose laurate as an example

1.5 References chapter 1

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2 Establishing a deep eutectic solvent system for the enzymatic biosurfactant synthesis

This chapter is partially based on the following publication

Sustainable enzymatic synthesis of glycolipids in a deep eutectic solvent system

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Journal of Molecular Catalysis B: Enzymatic

Volume 133, pages 281-287

Available online: 24 January 2017

DOI: 10.1016/j.molcatb.2017.01.015

Authors' contribution to this publication

Sascha Siebenhaller designed the study, performed synthesis reaction and purification, analyzed the results and wrote the manuscript.

Claudia Muhle-Goll and **Burkhard Luy** performed and helping to analyze the NMR. Write and proof-read parts of the NMR sections.

Frank Kirschhöfer and **Gerald Brenner-Weiss** performed the ESI-ToF experiments. Write and proof-read parts of the ESI-ToF sections.

Ekkehard Hiller, **Michael Günther**, **Steffen Rupp** and **Susanne Zibek** performed the TLC-MALDI-ToF experiments. Proofread the manuscript.

Christoph Syldatk supervised the project and proofread the manuscript.

2.1 Abstract

The first task of the project was to find a new and sustainable reaction system for the enzymatic catalyzed synthesis of glycolipids and sugar alcohol esters. For this, the usage of hazardous and environmental harmful organic solvents should be avoided. Therefore, this chapter describes the establishment of a novel deep eutectic solvent reaction system. This new DES is based on choline chloride and a sugar, respectively a sugar alcohol, which acts as the hydrogen bond donor.

In total, five various sugars, three sugar alcohols and one anhydrosugar were used for the formation of a DES, which acts as a reaction media for the enzymatic synthesis of surfactants in combination with up to nine different fatty acids. The proposed lipase catalyzed acylation of the different sugars and sugar alcohols, which are substrate and part of the reaction media at the same time, were detected by thin-layer chromatography and mass determination experiments. As an example for all synthesized surfactants, fragments and *m*/*z* signals of the expected glycolipid arabinose laurate were detected and its chemical structure was elucidated via NMR. Besides that, for the first time an anhydrosugar based DES was formed and a successful enzymatic synthesis was performed in it.

2.2 Materials and Methods

2.2.1 Materials

Lipase B from *Candida antarctica*, immobilized on acrylic resin (iCalB), choline chloride (98 %) and D(+)-Mannose (99 %) were purchased from Sigma Aldrich (Sigma Aldrich, St. Louis, USA). $CDCL_3$ (99.8 %) and acetone-d_6 (> 99.9 %) were from Eurisotop (Gif sur Yvette Cedex, France). All other utilized sugars and sugar alcohols were purchased from Carl Roth (Carl Roth KG, Karlsruhe, Germany). Levoglucosan was purchased from chemPUR (chemPUR GmbH, Karlsruhe, Germany). All fatty acid esters were purchased from Tokyo Chemical Industry Co. Ltd (TCI-Europe, Zwijndrecht, Belgium). All solvents were purchased in the highest purity available and used without further treatment.

2.2.2 Methods

2.2.2.1 Preparation of deep eutectic solvents

All DES for the enzymatic synthesis of biosurfactants were prepared by mixing equimolar ratios of CC with one of the sugars or sugar alcohols. The mixtures were constantly stirred and heated at 100 °C until a colorless liquid appeared.

Further, different ratios of CC and the second component were tested if they will form a DES.

2.2.2.2 Synthesis of glycolipids in DES

The reactions listed in Table 2.1 were performed by adding 100 mg of iCalB and 1.5 mmol of the fatty acid to 3.5 mL of the corresponding DES in a glass tube. The synthesis reaction ran up for 72 h at 1.500 rpm and 70 °C in an orbital shaker. Negative controls in all DES were performed without iCalB or a fatty acid. Every 24 h, samples from each reaction were taken to follow the formation of sugar and sugar alcohol esters via TLC.

Table 2.1: All accomplished synthesis reactions are marked with a cross (x).

	Vinyl hexanoate	Vinyl octanoate	Methyl octanoate	Vinyl decanoate	Methyl decanoate	Vinyl laurate	Methyl laurate	Vinyl palmitate	Methyl palmitate
Arabinose	x	x	x	x	x	x	x	x	x
Xylose	x	x	x	x	x	x	x	x	x
Glucose	x	x	x	x	x	x	x	x	x
Mannose	x	x	x	x	x	x	x	x	x
Rhamnose	x	x	x	x	x	x	x	x	x
Levoglucosan	-	-	-	-	-	x	-	-	-
Arabitol	x	-	-	-	-	x	x	-	-
Xylitol	x	-	-	-	-	x	x	-	-
Sorbitol	x	-	-	-	-	x	x	-	-

The presented transesterification (Figure 2.1) shows the synthesis of arabinose laurate. Theoretically, it might be possible that all other C-atoms of the sugar are acylated as well as the formation of di- or polyacylated sugars. The figure also applies to reactions with sugar alcohols.

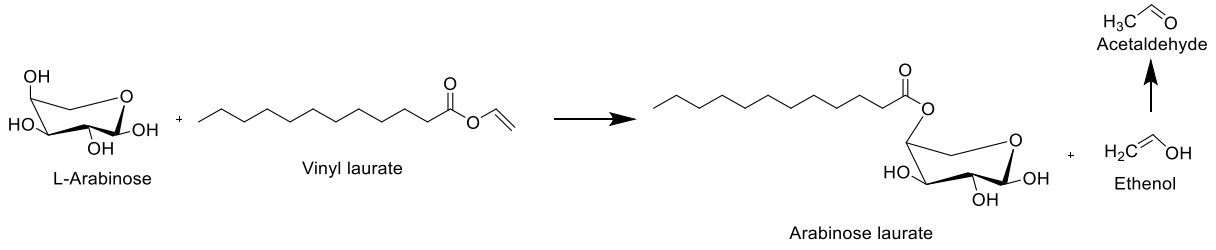

Figure 2.1: Transesterification between arabinose and vinyl laurate which leads to the formation of arabinose-4-*O*-laurate and ethenol. Ethenol will tautomerize to acetaldehyde and evaporates, pushing the reaction forwards. Instead of arabinose all other choosen sugars or sugar alcohols can be used.

2.2.2.3 Extraction of formed products

For qualitative analysis, and monitor the synthesis progress via thin-layer chromatography (TLC), samples from all reactions were extracted. For this, 150 µL of ddH_2O was heated to approximately 70 °C and a 150 µL DES sample from the reaction mixture was added. It was then shaken at 70 °C at 1.500 rpm for several minutes until each DES was fully dissolved. By adding 350 µL of ethyl acetate, one extraction step was performed under rigorous shaking for 45 s. After phase separation, the ethyl acetate phase, which contains the glycolipids, was taken and analyzed. The immobilized enzyme will stay in the aqueous phase.

2.2.2.4 Qualitative detection of sugar and sugar alcohol esters via thin-layer chromatography

For the thin-layer chromatography, 10 µL of the extracted samples were spotted onto a 60 Å silica gel TLC plate (Alugram Xtra SIL G, Macherey-Nagel GmbH & Co.KG, Düren, Germany). To separate the synthesized compounds, a mobile phase consisting of chloroform:methanol:acetic acid (65:15: 2 or 35:1:1, by vol.) was used. The separated compounds were visualized by dipping the TLC plate into an anise aldehyde solution (anise aldehyde:sulfuric acid:acetic acid; 0.5:1:100, by vol.) followed by heating under a 200 °C air flow for 5 min.

2.2.2.5 Determination of the masses via thin-layer chromatography coupled matrix-assisted laser desorption/ionization time of flight

With the TLC-MALDI-ToF technique it is possible to determine the mass of the product spots on a TLC plate (Fuchs et al., 2007). Two identical replicates of a TLC plate (5 x 7.5 cm HPTLC plate, silica gel 60, Merk, Darmstadt, Germany) were made. 4 µL of a 25 mg/mL solution of the extracted sample was sprayed as a 6 mm line on the TLC plate (ATS4, CAMAG, Switzerland) and was separated with the solvent system chloroform : methanol : acetic acid (35 : 1 : 1, by vol.). One plate got dyed with an anise aldehyde solution. On the second plate, a drop of a polymer standard (Polypropylenglycol 425; 1:100) was placed in a corner, afterwards the plate was dipped for 2 s in a matrix solution (200 g/L dihydroxybenzoic acid in 90 % acetonitrile, 1.15 g/L diammonium phosphate, 0.2 g/L octyl β-D-glucopyranoside, 0.1 % trifluoroacetic acid). Subsequently, the plate was set onto the MTP TLC Adapter for a Bruker Ultraflex II ToF/ToF (Bruker AG, Rheinstetten, Germany). The dyed plate served as a template for a coordinate system for the laser pulses (25 kV ion source, offset 63 %, range 15 %, positive mode). Every mm of the coordinate system measurements were made at three juxtaposed positions. The measured dataset was analyzed by the instrument software flexControl 3.0.

2.2.2.6 Purification and fractionation of arabinose laurate extracts by medium pressure liquid chromatography

To determine the exact mass of arabinose laurate as a representative surfactant via ESI-ToF and for structural characterization via tandem mass spectrometry and NMR, a purification step was accomplished by medium pressure liquid chromatography (SepacoreX50, Büchi Labortechnik GmbH, Flawil, Switzerland). Multiple extracts from ten identical synthesis reactions were combined, concentrated (Heidolph Laborota 4000, Schwabach, Germany) and purified through a column (40-63 µm particle size, 60 Å pore size, 150 mm column length, 12 mm column diameter, Büchi Labortechnik GmbH, Flawil, Switzerland). A chloroform:hexan (4:1, by vol.) / methanol solvent system was used at a flow rate of 5 mL/min (5 min 0 %, in 40 min to 10 %, 5 min 10 %, in 3 min to 20 %, in 3 min to 100 % and 4 min 100

% methanol). The eluate was collected in 5 mL fraction size and analyzed via TLC. Combined fractions 50 to 57 were used for further analysis.

2.2.2.7 Verification of the masses and structure determiantion via electro spray ionization quadrupole time of flight mass spectrometry (ESI-Q-ToF)

Mass determination of the extracted compounds were performed using an ESI-Q-ToF system (Q-Star Pulsar i, AB SCIEX, Darmstadt, Germany) equipped with an electrospray ionization (ESI) source operating in the positive mode within a mass range from *m*/*z* 50 to *m*/*z* 800 using the activated "enhance all" setting. Prior to analysis, the samples were diluted 1:5 in methanol:10 mM ammonium acetate (1:1, by vol.) and were continuously infused via a syringe pump at a flow rate of 10 µL/min.

The ion source voltage was set at 4,800 V. The declustering potential was adjusted at 30 V and focusing potential at 100 V. MS/MS-experiments were carried out in the product ion scan mode at collision energy of 20 V, and the MS spectra were recorded in the mass range from *m*/*z* 50 to *m*/*z* 400. In all experiments, nitrogen gas 5.0 was used as nebulizer, curtain and collision gas.

Data acquisition and processing were performed using the Analyst QS 1.1 software (AB SCIEX, Darmstadt, Germany).

2.2.2.8 Structural elucidation via nuclear magnetic resonance spectroscopy

For NMR spectroscopy, 14 mg of purified fractions 50-57 was dissolved in 0.6 mL $CDCl_3/d_6$-acetone (4:1, by vol.). 1D ^1H NMR spectroscopy and 2D ^1H-^1H correlation spectroscopy (COSY), total coherence transfer spectroscopy (TOCSY), nuclear Overhauser effect spectroscopy (NOESY), ^1H-^{13}C heteronuclear single quantum coherence spectroscopy (HSQC), and heteronuclear multiple-bond correlation spectroscopy (HMBC) were recorded on a Bruker AVANCE II+ 600-MHz spectrometer (Bruker AG, Rheinstetten, Germany) equipped with a BBI probe head. Spectra were analyzed with Topspin 3.2 (Bruker AG) and SpinWorks 3.1.8 software (Marat). Intensities were measured from a 1D ^1H spectrum acquired with a sixteen scan and four dummy scans. Chemical shifts are referenced to the ^1H and ^{13}C resonance trimethylsilan.

2.3 Results and Discussion

2.3.1 Formation of deep eutectic solvents

Various sugar- and sugar alcohol ratios were tested to investigate in which molar ratios the formation of a CC based DES is possible (Table 2.2).

Table 2.2: The table shows all investigated combinations for the formation of a DES. Combination which were marked with a "x" indicate the successful formation of a DES under the mentioned conditions; combinations with a "–" will not form a DES. With mannitol, it is possible to form a DES by increasing the temperature to 130 °C. Combinations which were indicated with "n.t." have not been tested.

CC / Carbohydrate	3:1	2:1	1:1	1:2	1:3
Arabinose	n.t.	x	x	x	n.t.
Xylose	n.t.	x	x	x	n.t.
Glucose	n.t.	x	x	x	n.t.
Mannose	n.t.	x	x	x	n.t.
Rhamnose	n.t.	x	x	x	n.t.
Levoglucosan	n.t.	n.t.	x	n.t.	n.t.
Arabitol	n.t.	x	x	x	n.t.
Xylitol	n.t.	x	x	x	n.t.
Sorbitol	x	x	x	x	x
Inositol	-	-	-	-	-
Mannitol	x*	x*	x*	-	-

The results show, that nearly all tested combinations were able to form a DES. Only by using the sugar alcohols inositol and mannitol, the formation of a DES under the given parameters was not possible. To form a DES with mannitol, it was necessary to increase the temperature to 130 °C, with inositol this did not lead to the desired result. Our findings are consistent with DES described in literature (Dai et al., 2013). Next to CC, it is possible to use other quaternary ammonium salts such as betaine to form a DES (Aroso et al., 2017). By using betaine, it is possible to form a DES in the molar ratio of 9:1:1 with inositol and raffinose (Dai et al., 2013). It has not been clarified yet, why it is not possible to form a DES with inositol as sole hydrogen bond donor.

2.3.2 Sugar- and sugar alcohol ester detection via thin-layer chromatography

To monitor the possible formation of glycolipids and alditollipids, all extracts were analyzed via TLC in the two described mobile phases. The separated compounds were visualized and evaluated. In all negative controls one colored spot appeared for the corresponding sugar with the lowest retention factor (RF) < 0.08.

The extracts of the synthesis reactions of the three hexoses, glucose, mannose and rhamnose showed various potential product spots, in combination with all used fatty acids. Exemplary for the hexoses, the DES CC:mannose (Figure 2.2), in combination with vinyl laurate showed six spots with RFs 0.78; 0.45

(double-spot); 0.37; 0.25; 0.16 and 0.06 when using the mobile phase 65:15:2. TLCs with all other tried fatty acid variants from C6 to C14 showed nearly the same spots.

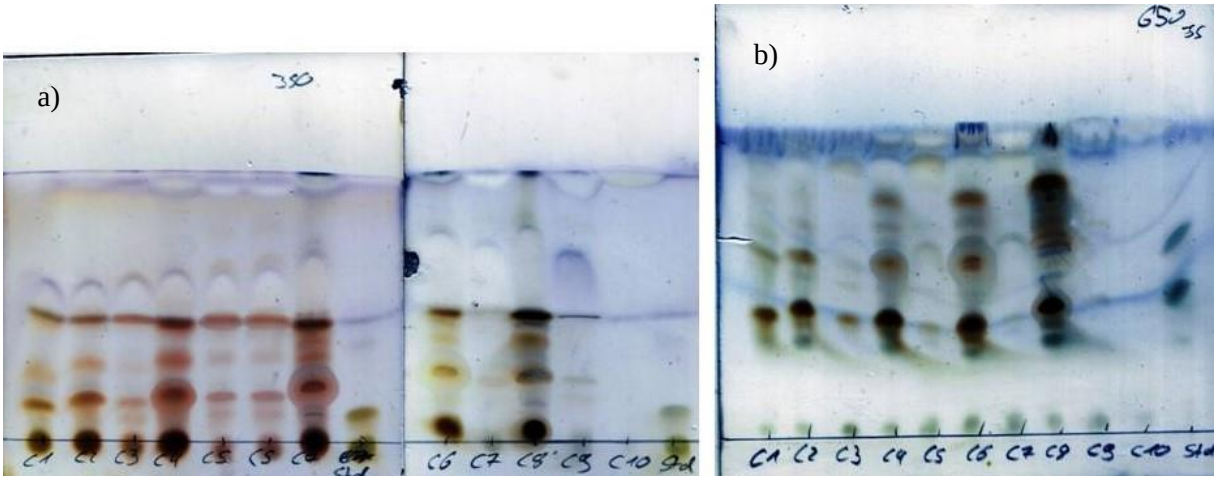

Figure 2.2: Thin-layer chromatography of synthesis products from the enzymatic reaction of various fatty acids by iCalB in a DES reaction system of CC:mannose. As mobile phase of plate a) mixture 65:15:2, for plate b) 35:1:1 was used. Both plates were dyed with anise aldehyde solution. Colored spots indicate the formation of various sugar esters. 1 = vinyl hexanoate, 2 = vinyl octanoate, 3 = methyl octanoate, 4 = vinyl decanoate, 5 = methyl decanoate, 6 = vinyl laurate, 7 = methyl laurate, 8 = vinyl palmitate, 9 = methyl palmitate, 10 = negative control without enzyme, Std = lab intern rhamnolipid standard.

Furthermore, all separated spots from the pentoses synthesis extracts showed product spots, too. As an example for all tested reactions with pentoses, the combination of CC:arabinose with vinyl laurate was used (Figure 2.3). After dyeing, spots with RFs of 1; 0.88 (double spot); 0.51; 0.10 and 0.02 occurred.

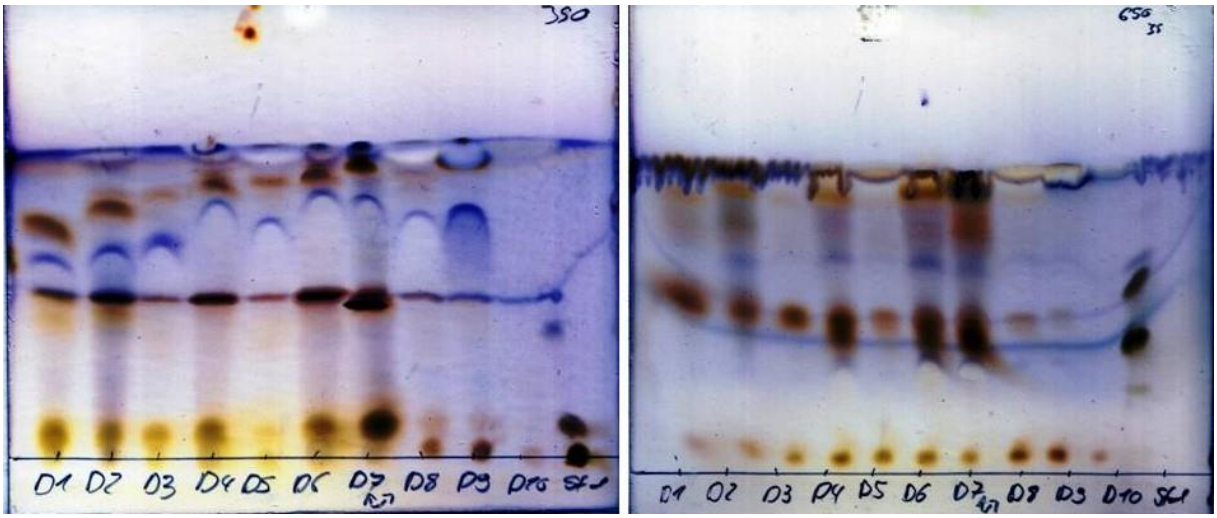

Figure 2.3: TLC of synthesis products from the enzymatic reaction of various fatty acids by iCalB in a DES reaction system of CC:arabinose. As mobile phase of plate a) mixture 65:15: 2, for plate b) 35:1:1 was used. Both plates were dyed with anise aldehyde solution. Colored spots indicate the formation of various sugar esters. 1 = vinyl hexanoate, 2 = vinyl octanoate, 3 = methyl octanoate, 4 = vinyl decanoate, 5 = methyl decanoate, 6 = vinyl laurate, 7 = methyl laurate, 8 = vinyl palmitate, 9 = methyl palmitate, 10 = negative control without enzyme, Std = lab intern rhamnolipid standard.

The observed spots in each tested reaction indicate that iCalB was active in all sugar-based DES. Additionally, the number of spots can be a hint for the suspected formation of single- and poly-acylated

sugars. When mannose, glucose or rhamnose were part of the DES, the RF-values of the separated compounds were strongly affected by the sugar. For each fatty acid used, the RFs of the separated compounds are nearly the same. If arabinose or xylose were the substrate for the reaction, the RF-values would increase with the chain length of the corresponding fatty acid. This can be explained by the higher hydrophobicity of the longer fatty acid chain which results in a faster movement in the mobile phase.

For the first time it has been reported that a DES based on CC and an anhydrosugar was formed. Further, this DES with the anhydrosugar levoglucosan was used as a media and substrate for the synthesis reaction with vinyl laurate; more reactions were not accomplished due to the high costs for levoglucosan. The components contained in the extract have been separated via TLC by the usage of the running agent 35:1:1. After visualization, there were four possible product spots with the RF values 0.87; 0.76; 0.57; 0.39, as well a levoglucosan spot with an RF 0.04 (Figure 2.4 a). This result shows, that it is possible to synthesize glycolipids with levoglucosan as polar head.

Synthesis in alditol-based DES show a different staining behavior and spot pattern. Synthesis products does not show the yellow color when using anise aldehyde solution, but rather pale blue spots. In these DES, the RF values are nearly the same as in sugar-based DES when using fatty acid vinyl esters. As an representative, formed synthesis products of a reaction in a DES of CC:xylitol were separated via TLC and subsequently visualized (Figure 2.4 b). The TLC show is showing a double spot in lane 1 and 3 with an RF of 0.68, indicating the formation of an alditollipid. As fatty acid, vinyl hexanoate, respective vinyl laurate was used. When using a fatty acid methyl ester (lane 2 and 4) or octanoic acid (lane 5), no product spots are visible. All lanes show a spot at RF 0.16, indicating for xylitol as part of the DES. The other lanes (6-8) are negative controls. Results with arabitol and sorbitol are similar to the shown results.

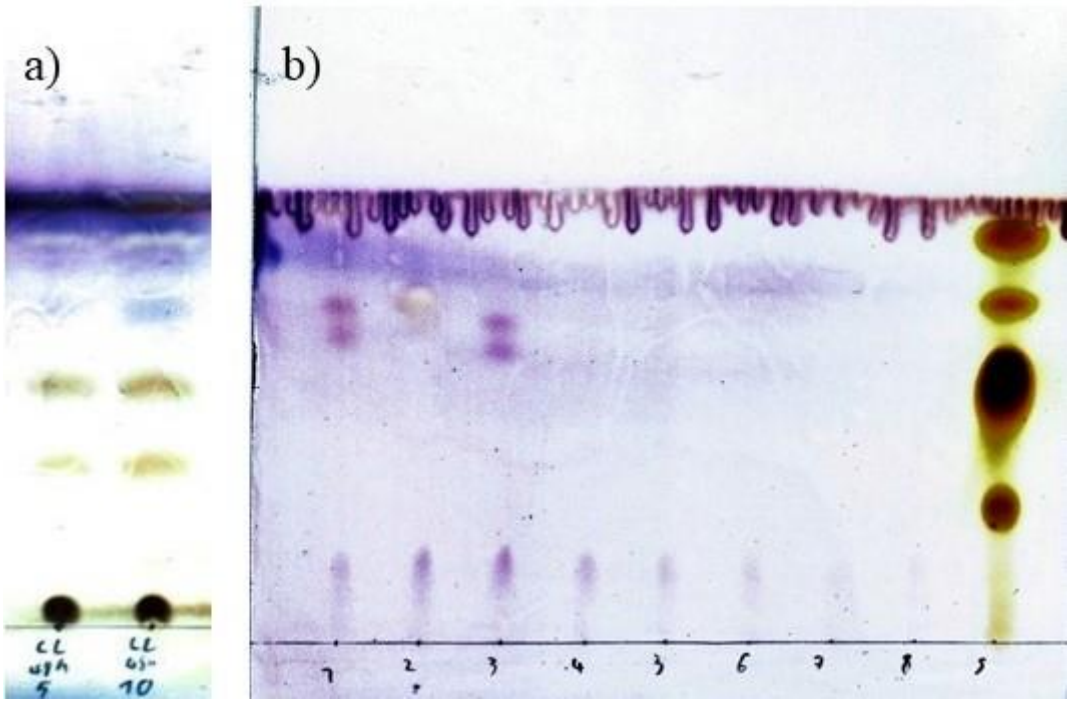

Figure 2.4: a) The TLC shows the formed and separated products of a synthesis reaction in a DES based on CC:Levoglucosan with vinyl laurate after visualization. As mobile phase, the 35 : 1 : 1 mixture was used. b) The extract of a synthesis reaction in a DES based on CC:xylotol was separated (65:15:2) and visualized. The following fatty acid esters were used a substrate: 1 = vinyl hexanoate, 2 = methyl hexanoate, 3 = vinyl laurate, 4 = methyl laurate, 5 = octanoic acid, 6-8 = negative controls, S = lab intern rhamnolipid standard.

Besides potential product spots, other spots can possible occur in visualized TLCs due to of the usage of the crude extract or by the formation of side-products like sugar acetals.

The first TLC results indicate, that sugars and fatty acid vinyl esters are more favored by the used lipase under these conditions. When using a sugar alcohol or a fatty acid methyl ester, spots seems to be much weaker. But this are just preliminary results and have to be investigated further with kinetic studies. Both side-products, acetaldehyde and methanol, should evaporate at 70 °C pushing the reaction towards acylation. In general, the synthesis reactions with the fatty acid vinyl esters showed stronger spots than the reactions with fatty acid methyl esters. A reason for the weaker spots could be, that the formed methanol had a negative influence on the activity of iCalB (Bajaj et al., 2010; Bélafi-Bakó et al., 2002) (Figure 2.5).

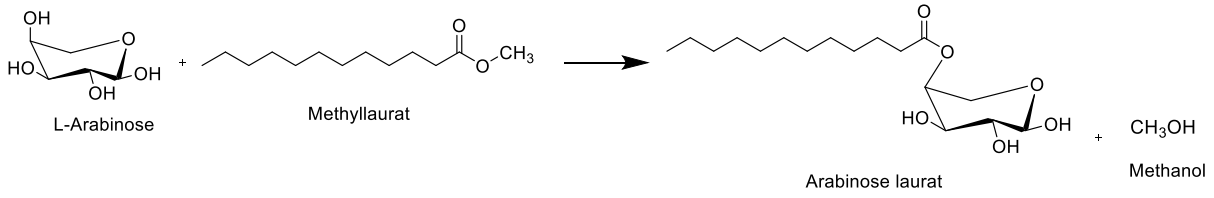

Figure 2.5: Transesterification between arabinose and methyl laurate which leads to the formation of arabinose laurate and methanol. Methanol can evaporate, pushing the reaction forwards. But it can also damage the used lipase, which leads to a lower transesterification rate.

In spite of the benefits of the used DES-based system, the high viscosity of the DES can cause mass-transfer problems which can lead to lower yields. To avoid this issue, a thorough and constant mixing of all components must be ensured.

All other TLC of reactions in CC:Glucose and CC:Rhamnose are shown in Appendix 1 and Appendix 2.

2.3.3 Mass determination of crude synthesis extracts via TLC-MALDI-ToF

A TLC-MALDI-ToF was performed to further analyze the product spots and to confirm the formation of glycolipids and alditollipids. With this technique it is possible to assign a product spot to a detected *m*/*z* mass. Because of the high number of accomplished synthesis reactions, only extracts from the synthesis of two hexoses (glucose and mannose), two pentoses (arabinose and xylose) and three sugar alcohols (sorbitol, arabitol and xylitol) with vinyl laurate were used for the TLC-MALDI-ToF. The chosen compounds serve as representatives for other synthesized surfactants.

The formation of glycolipids as suggested in Figure 2.1 leads to arabinose laurate, but theoretically more than one laurate molecule can be transacylated to the arabinose. The calculated masses of all possible acylated sugar laurates are shown in Table 2.3.

Table 2.3: Calculated neutral, monoisotopic molar masses of the possible synthesized glycolipids and alditollipids.

Type of glycolipid	Calculated mass [Da]	Abbreviation	Type of alditollipids	Calculated mass [Da]	Abbreviation
Hexose laurate	362.23	M	Sorbitol laurate	364.25	MA
Hexose di-laurate	544.40	M_2	Sorbitol di-laurate	546.41	MA_2
Hexose tri-laurate	726.56	M_3	Sorbitol tri-laurate	728.58	MA_3
Hexose tetra-laurate	908.73	M_4	Sorbitol tetra-laurate	910.75	MA_4
Pentose laurate	332.22	*M*	Pentose laurate	334.24	*MA*
Pentose di-laurate	514.73	M_2	Pentose di-laurate	516.41	MA_2
Pentose tri-laurate	696.55	M_3	Pentose tri-laurate	698.57	MA_3
Pentose tetra-laurate	878.72	M_4	Pentose tetra-laurate	880.74	MA_4

In all measurements, sodium adducts of sugar- and sugar alcohol esters were identified. The detected *m*/*z* 538.02 respectively *m*/*z* 537.97 confirm the formation of pentose di-laurates as the sodium adducts of arabinose and xylose (M_2+Na^+). Furthermore, as shown in Table 2.4 the *m*/*z* 719.57 and *m*/*z* 719.37 as well as *m*/*z* 901.48 and *m*/*z* 901.42 verify the formation of pentose tri- and tetra-laurates (M_3+Na^+ and M_4+Na^+). The *m*/*z* of M_2+Na^+ and M_3+Na^+ were detected for the hexoses, glucose and mannose. Further, for mannose the sodium adduct of mannose tetra-laurate with *m*/*z* 931.44 were detected, too.

When using alditols as part of a DES, the synthesis of alditollipids with iCalB under the given parameters is successful, too. As with the use of sugars, several alditol di-, tri- and tetra-laurates were detected (Table 2.4).

Additionally, several other signals were detected. Some *m*/*z*-values belong to the corresponding product with one- or more cleaved H_2O. Thus the *m*/*z* 661.61 should be an ionized arabinose- or xylose-tri-laurate - 2 H_2O. The same is true for the *m*/*z* 691.51 and *m*/*z* 873.72. A possible explanation for the other observed signals is the lack of a purification step of the used crude synthesis extracts.

Table 2.4: Detected *m*/*z* values of possible sugar- and alditol laurates via TLC-MALDI-ToF. Detected masses correspond to the respective sodium adduct.

Type of DES	Detected *m*/*z* signals	Possible correspondent
CC:Arabinose	538.02	$M_2 + Na^+$
	719.57	$M_3 + Na^+$
	901.48	$M_4 + Na^+$
CC:Xylose	537.97	$M_2 + Na^+$
	719.37	$M_3 + Na^+$
	901.42	$M_4 + Na^+$
CC:Glucose	567.88	$M_2 + Na^+$
	749.56	$M_3 + Na^+$
CC:Mannose	567.92	$M_2 + Na^+$
	749.42	$M_3 + Na^+$
	931.44	$M_4 + Na^+$
CC:Arabitol	539.09	$MA_2 + Na^+$
	721.21	$MA_3 + Na^+$
	903.33	$MA_4 + Na^+$
CC:Xylitol	539.29	$MA_2 + Na^+$
	720.96	$MA_3 + Na^+$
	919.45	$MA_4 + Na^+$
CC:Sorbitol	569.42	$MA_2 + Na^+$
	751.46	$MA_3 + Na^+$
	933.62	$MA_4 + Na^+$

These results show that iCalB is active in all seven tested DES and that it is possible to synthesize poly-acylated glycolipids and alditollipids in this DES based system. Since further projects will focus on the usage of sugars for the production of surfactants, no further experiments with sugar alcohols were carried out.

This MS method is only applicable for molecules with masses >500 Da due to matrix interactions. To determine the formation of sugar mono-laurates and to verify the results, purified extracts of arabinose laurate were used for further mass determination via ESI-Q-ToF.

All mass spectra are shown in Appendix 3 to Appendix 6.

2.3.4 Verification of the mass and structure elucidation via ESI-Q-ToF MS

The purified fraction 50-57 was used to confirm the formation of arabinose laurate glycolipids via ESI-Q-ToF MS (Appendix 7). This method provides a better determination of the accurate mass than the TLC-MALDI-ToF MS approach.

When comparing to the observed masses found by TLC-MALDI-ToF MS, the proposed formation of arabinose laurate could be clearly proved by the presence of the ion of *m*/*z* 350.225 ($M+NH_4^+$). All acquired *m*/*z* values are listed in Table 2.5.

Table 2.5: Observed *m*/*z* values during ESI-Q-ToF experiment. Masses compare to arabinose-4-O-laurate with a theoretical, neutral, monoisotopic molar mass of 332.219 Da (=M).

m/*z* value	Corresponding fragment
115.046	Arabinose + H^+ - 2 H_2O
315.193	$M+H^+$ - H_2O
350.225	$M+NH_4^+$

Additional MS/MS experiments were carried out to confirm the identity of the isolated compound, which should lead to specific fragment masses in case of arabinose laurate (Figure 2.6).

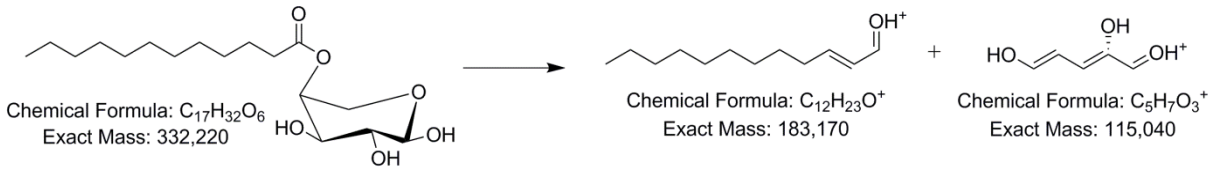

Figure 2.6: Possible MS/MS fragmentation of arabinose laurate during ESI-Q-ToF MS/MS measurement.

The product ion scan spectrum of the ion of *m*/*z* 315 showed high-abundance fragmented ions (see MS/MS-spectra in Appendix 8). The fragmentation patterns are shown in Table 2.6. All detected *m*/*z* signals are logical fragments of arabinose laurate.

Table 2.6: MS/MS-experiment, observed *m*/*z* values by a product ion scan of *m*/*z* = 315.

m/*z*	Corresponding fragments
97.042	Arabinose + H^+ - 3 H_2O
115.049	Arabinose + H^+ - 2 H_2O ($C_5H_7O_3^+$)
183.185	Tail of the fatty acid ($C_{12}H_{23}O^+$)
297.205	$M+H^+$ - 2 H_2O
315.206	$M+H^+$ - H_2O

The ESI-Q-ToF experiments verify the formation of arabinose laurate during a synthesis with iCalB in a DES consisting of CC:Ara. These results should stand as a representative example for all other tested DES in combination with the tested fatty acid ester. MS experiments from fraction 30-34 show the formation of arabinose di-laurates as well (data not shown).

For full elucidation of the correct chemical structure of the obtained arabinose laurate fraction 50-57 was analyzed using NMR.

2.3.5 Configuration of arabinose laurate structure by NMR

Three major carbohydrate systems were identified through analysis of ^{1}H COSY, ^{13}C HSQC and ^{13}C HMBC spectra. Starting with the anomeric protons, the rings could be traced in ^{1}H COSY and ^{13}C HMBC spectra. One of these, arabinose C (Table 2.7), corresponded to the free arabinose, which is part of the deep eutectic solvent. The other two systems correspond to acylated arabinoses on the basis of cross peaks of an arabinose proton with lipid carbonyls in the $^{1}H^{13}C$ HMBC spectrum (Figure 2.7). Arabinose A is acylated on C4 and arabinose B on C3. The lipid chains had identical resonances. Alternatively, arabinose A could be acylated on both C4 and C3, and arabinose B could be the anomer of arabinose C, since overlap in proton resonances prevented unambiguous assignment of the corresponding cross peak in the $^{1}H^{13}C$ HMBC. But since, the results of the ESI-ToF experiments showed that in fraction 50-57 only arabinose mono-laurates and no di-acylated arabinose occurred, the latter possibility is not highly likely. This suggests that the used lipase is not very specific, so other configurations are possible, too. Reaction with ethenol, which rearranges to acetaldehyde resulted in acetal formation between the sugar and the acetaldehyde. Several arabinose systems that vary in the position of the acetaldehyde attachment can be distinguished in the spectra.

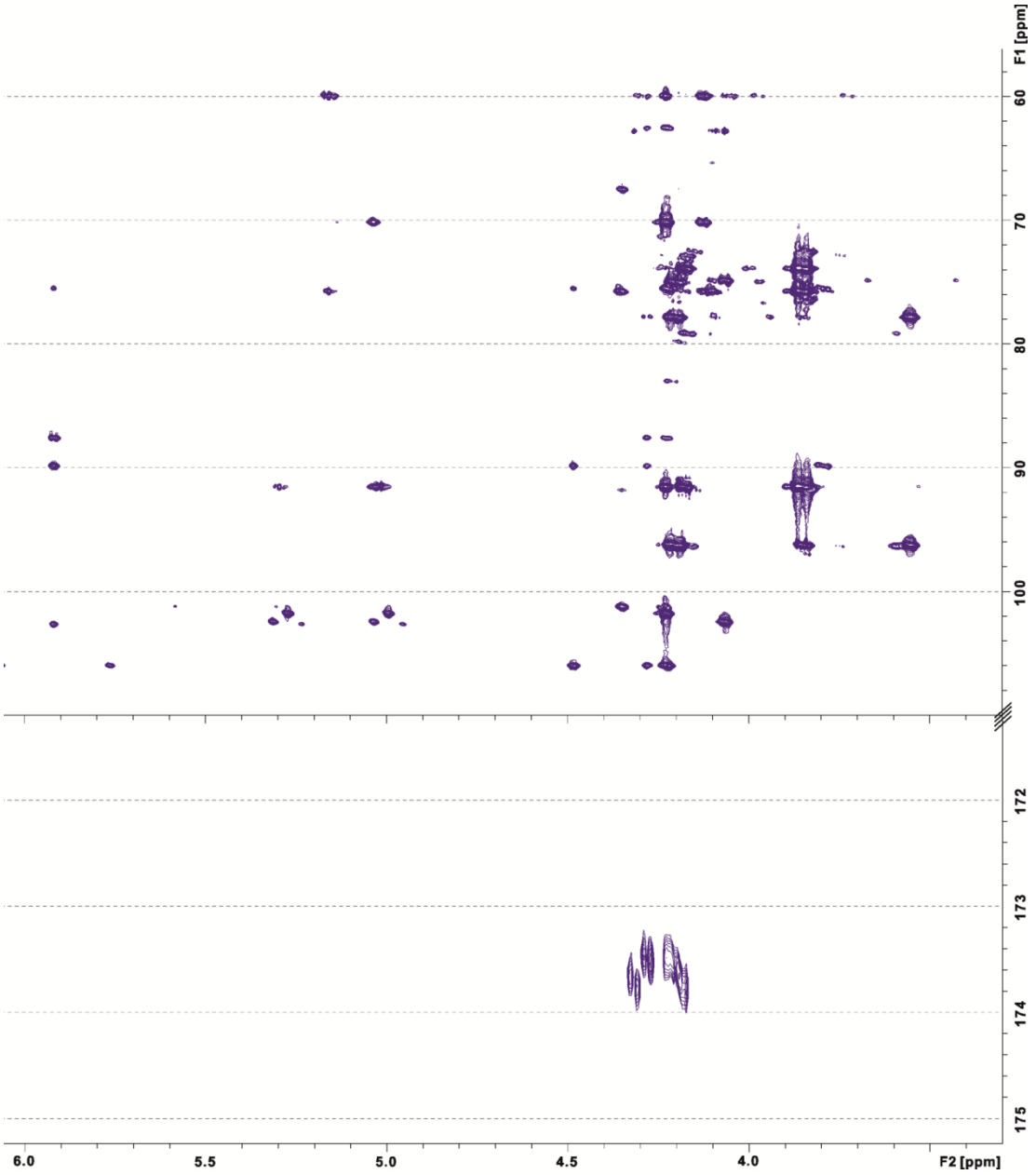

Figure 2.7: ^{1}H ^{13}C HMBC of fraction 50-57 with cross-peaks of arabinose protons with lipid carbonyls.

Table 2.7: Chemical shifts of the main products present in fraction 50-57. d = doublet; t = triplet; m = multiplet; n.d. = not determinable.

arabinose A	C Shift [ppm]	H Shift [ppm]	Multiplicity	Coupling [Hz]
─C^{1}H─O─	106.0	5.92	d	4.25
─C^{2}H─	87.7	4.48	dd	4.25, <3 Hz
─C^{3}H─ (*acylated C' 173.48)	89.9	4.22	Overlap	-
─C^{4}H─ (acylated C' 173.49)	75.5	4.28	Overlap	-
─C^{5}H─	62.6	3.80, 3.71	m	6.5, 10.8, 9.9

arabinose B	C Shift [ppm]	H Shift [ppm]	Multiplicity	Coupling [Hz]
─C^{1}H─O─	91.6	5.16	Overlap	-
─C^{2}H─	75.7	4.23	Overlap	-
─C^{3}H─ (*acylated C' 173.48)	70.1	3.77	Overlap	-
─C^{4}H─	73.8	4.13	ddd	2.0, 3.1, 6.2
─C^{5}H─	59.8	4.16, 3.85	Overlap	-

arabinose C	C Shift [ppm]	H Shift [ppm]	Multiplicity	Coupling [Hz]
─C^{1}H─O─	96.3	4.56	d	6.5
─C^{2}H─	74.9	3.56	dd	6.5, 12.9
─C^{3}H─	77.9	4.07	dd	6.8, 12.9
─C^{4}H─	75.0	4.09	Overlap	-
─C^{5}H─	62.8	4.20, 3.85	Overlap	-

* Possible acylation site

Laurate	C Shift [ppm]	H Shift [ppm]	Multiplicity	Coupling [Hz]
O=C^{1}─OH	173.48			
─C^{2}H$_2$─	34.2	2.34	dd,dd	2,0, 7.6, 7.6
─C^{3}H$_2$─	24.7	1.62	Overlap	-
─C^{4}H$_2$─	29.3	1.30	Overlap	-
─C^{5}H─	29.2	1.26	Overlap	-
─C^{6}H─	29.2	1.26	Overlap	-
─C^{7}H$_2$─	29.2	1.26	Overlap	-
─C^{8}H$_2$─	29.2	1.25	Overlap	-
─C^{9}H$_2$─	29.1	1.29	Overlap	-
─C^{10}H$_2$─	31.8	1.26	Overlap	-
─C^{11}H$_2$─	22.6	1.26	t	7.1
─C^{12}H$_3$	14.0	0.88	t	6.8

2.4 Conclusions

A wide variety of potential substrates for the enzymatic glycolipid production in DES were tested by screening the conversion of sugars (three hexoses and two pentoses), the anhydrosugar levoglucosan and three sugar alcohols in combination with up to nine different fatty acids. The described DES-based solvent system acts simultaneously as substrate and solvent and is suitable for the enzymatic synthesis of novel tailor-made glycolipids and alditollipids. The formation of surfactants by a lipase-catalyzed acylation of the used substrates were detected by TLC-MALDI-ToF. Since the focus lies on the usage of sugars, no further experiments with sugar alcohols have been carried out.

To verify the results and for a structural elucidation, arabinose laurate was chosen as a representative glycolipid. With ESI-Q-ToF and further MS/MS experiments as well as multidimensional NMR experiments, the successful synthesis of arabinose laurate was confirmed.

With this reaction system, it was possible for the first time to form a DES based on CC and levoglucosan, and to successfully use it with iCalB for an enzymatic synthesis reaction. This finding should help to create a novel class of glycolipids with more beneficial characteristics.

It was observed via TLC that the formation of glycolipids with fatty acid vinyl esters as substrate is more effective than with fatty acid methyl esters. The possible reason for this is, that the methanol, which is split of during the transesterification, has negative influences on the used iCalB.

It is also interesting that the iCalB is not specific for one glycolipid product. Besides to sugar monolaurates, many poly-acylated sugars were formed during synthesis and were detected via TLC-MALDI-ToF and ESI-Q-ToF. The enzymatic synthesis of tri-and tetra acylated sugars has not been described before. Moreover, the results of ESI-Q-ToF and NMR analysis suggested that the configuration of the acylated arabinose laurates varies, which leads to glycolipids with potential different characteristics

2.5 References Chapter 2

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3 Time-depending formation and properties of arabinose laurate

This chapter is prepared for submission as a short communication

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Authors' contribution to this manuscript

Sascha Siebenhaller designed the study, performed experiments, analyzed the results and wrote the manuscript. Supervised Maria Velaz Martin.

Maria Velaz Martin performed experiments, evaluated results and proofread the manuscript.

Marius Henkel and **Rudolf Hausmann** helped to perform the HPTLC measurements, proofreading the manuscript.

Alexander Wilsdorf and **Rumen Krastev** helped to determine the cmc-value.

Christoph Syldatk supervised the project, provided scientific input and proofread the manuscript.

3.1 Abstract

After establishing a sugar based DES reaction system and verifying the formation of glycolipids, in particular of arabinose laurate, this chapter focuses on the time-depending synthesis and reaction yields of arabinose laurate as well as its surface properties. The first aim of this study was to determine the best suitable ratio of choline chloride and arabinose. The second goal is the determination of the optimal water content and water activity for the DES reaction media in order to optimize the enzymatic arabinose laurate synthesis. The third parameter investigated is the synthesis duration. To determine the time-dependent formation of arabinose laurate and di-laurate and the yields, several reactions were performed and analyzed via high performance thin-layer chromatography. The last aim was to determine important surface properties of purified arabinose laurate via a Wilhelmy Plate Tensiometer. Therefore, the critical micelle concentration of the surfactant as well as the ability to reduce the surface tension of a water to air interface were determined to compare it with other available glycolipids.. Further, the surface excess concentration were determined, too.

It was observed that the reaction yields are strongly dependent on the water content and activity of the DES. Small amounts of water increase the water activity and can more than double the yield of formed arabinose laurate to approximately 50 % in 48 h. Further, water concentrations above 8 % slightly decreased the yields. Vice versa, the occurrence of water inhibited the formation of arabinose di-esters, so it is possible to steer the product formation into a desired direction. Additionally, it was detected that the impact of the DES ratio did not influence the yields strongly and that most of the glycolipids were formed during the first 24 h. Afterwards, most reactions reached a product plateau. Arabinose laurate seems to be an appropriate surfactant due to its ability to lower the surface tension of demineralized water from 72 mN/m to 23 mN/m with a CMC of 530 mg/L. With these properties, it might be an interesting surfactant for various water based applications. Surface properties of arabinose di-laurate could not be determined due to too low yields after purification.

3.2 Materials and Methods

3.2.1 Materials

Lipase B from *Candida antarctica*, immobilized on acrylic resin (iCalB) and choline chloride (98 %) were purchased from Sigma Aldrich (Sigma Aldrich, St. Louis, USA). Arabinose was purchased from Carl Roth (Carl Roth GmbH, Karlsruhe, Germany), vinyl laurate from Tokyo Chemical Industry Co. Ltd (TCI-Europe, Zwijndrecht, Belgium). TLC plates were purchased by Merck-Millipore (Merck KGaA, Darmstadt, Germany). All solvents were purchased on the highest purity and used without further treatment.

3.2.2 Methods

3.2.2.1 Preparation of deep eutectic solvents

All used DES are based on choline chloride (CC) and arabinose (Ara). Used CC was dried for at least 24 h under vacuum in a desiccator, equipped with dry silica gel, before use. To form a DES, three different molar ratios of CC and Ara (2:1; 1:1 and 1:2) were mixed. Subsequently, to 10 g of the mixture 200 µL, 400 µL, 800 µL or 1,200 µL water was added to gain DES with five different ratios of water (0 %, 2 %, 4 %, 8 % and 12 %). After adding all components, the mixture was constantly stirred and heated up to 100 °C until a colorless liquid was formed.

3.2.2.2 Determination of the water activity

The water activity is an essential parameter for enzymatic reactions. Therefore, the water activity of all 15 DES were determined according to the manufacturer with a dew point hygrometer (AquaLab 4TE, Meter Group, Germany). All measurements were done in duplicates.

3.2.2.3 Production of arabinose laurate and arabinose di-laurate standards

To quantify the synthesized glycolipids arabinose laurate and arabinose di-laurate a pure standard of both components is necessary. For this, six synthesis reactions were performed as follows: 100 mg iCalB, 250 µL vinyl laurate and 2.25 g of a DES with a ratio of 1:1 and 0 % water were added in a 5 mL reaction vessel. The reaction proceeded for 48 h at 50 °C in a rotator with vortex mixer in program U2 at 50 rpm (neoLab, Heidelberg, Germany).

To extract the glycolipids, 2 mL of 70 °C hot water were added to solve the DES. Subsequently, all six solved DES were unified in a 50 mL Falcon-tube and 20 mL ethyl acetate were added. After 30 s of shaking, the glycolipid containing upper phase was transferred into a new vessel. The ethyl acetate extraction was repeated three times and extracts were unified. The combined extract was concentrated to

a final volume of 5 mL in a speed vac. Afterwards, a total volume of 400 µL was transferred as a thin line (17 cm wide, less than 2 mm thick) on a preparative TLC plate (PLC Kieselgel 60, 0.5 mm, 10 x 20 cm). For a migration distance of 7 cm, a mobile phase of chloroform:methanol:acetic acid (70:5:2, by vol.) was used. A 3 cm wide strip of the TLC plate was cut off and the formed arabinose esters were dyed by dipping the TLC plate into a thymol solution (2 g thymol, solved in 10 mL sulfuric acid and 190 mL ethanol) and developed under a 120 °C air stream for 5 min. This dyed stripe was used to detect the arabinose esters on the other part of the TLC plate. Arabinose laurate or di-laurate containing silica parts were scraped of separately (Figure 3.1). To gain sufficient amount of pure arabinose laurate and arabinose di-laurate, respectively, these steps were repeated three times.

Figure 3.1: TLC plate, from which arabinose laurate and di-laurate were scratched off.

Afterwards, all scratched-off silica with one kind of sugar esters was unified and 25 mL methanol were added to solve the glycolipids. After 5 h of stirring at room temperature, the silica was removed by filtration (nylon filter, 0.22 µm). Each extract was transferred into a pre-weighted round-flask and methanol was evaporated in a rotary evaporator at 45 °C under reduced pressure. Subsequently, the glycolipid containing round flasks were weighted again. The quantity of glycolipid was determined due to the weight differences of the flasks.

Finally, for each mg of arabinose laurate or, respective arabinose di-laurate, 1 mL of methanol was added to obtain a final concentration of 1 mg glycolipid per 1 mL solution.

3.2.2.4 Determination of the time-dependent formation and yields of arabinose esters in DES

To investigate the time-dependent formation of arabinose laurate and arabinose di-laurate in different DES with three molar ratios and five water contents, several enzymatic synthesis reactions were performed. Reactions were executed as follows: in 5 mL Eppendorf-tubes, 2 g of DES, 20 mg iCalB and 130 µL vinyl laurate were given. Afterwards, the synthesis reactions were performed in a rotator with vortex mixer in program U2 at 50 °C. For each molar ratio and water content, five identical approaches were performed. After 3 h, 7 h, 15 h, 24 h and 48 h, the reactions were stopped by adding 1 mL warm water to solve the DES. Subsequently, 2 mL ethyl acetate were added to extract the formed sugar esters and the tubes were vortexed for 30 s. The glycolipid containing organic phases were transferred into a

50 mL Falcon-tube. To extract over 90 % of all sugar esters, the extractions were performed six times (data not shown).

Each reaction was made as duplicate.

3.2.2.5 Determination the product formation via high-performance thin-layer chromatography

To determine the amount of synthesized arabinose laurate and di-laurate, a HPTLC analysis of all extracts were performed as duplicates. First, to solve potential product deposits in the Falcon-tube, 3 mL of methanol were added to all synthesis extracts. Afterwards, 1 mL of each extract was transferred to a HPLC-vial and placed in an automatic TLC sampler (ATS4, CAMAG, Berlin, Germany). 5 µl of each sample was sprayed as 5 mm long band on the TLC plate. Additionally, the arabinose laurate standard was sprayed 4-times (4 µL, 10 µL, 18 µL and 25 µL as 5 mm band) and the arabinose di-laurate standard 2-times (4 µL and 10 µL as 5 mm band) on the same plate as a defined standard to quantify the extracted samples.

After application of the samples, the TLC plate was transferred into an automatic developing chamber (ADC 2, CAMAG) and run with the following set-up: pre-drying of the plate, tank saturation for 8 min with 25 mL of running solvent, 2 min plate preconditioning time. At a migration distance of 70 mm the separation was stopped and the plate was dried for 5 min.

After separation of the compounds, the plate was dyed with thymol solution in an automatic dipping chamber (speed: 4, time: 2 s; Chromatogram Immersion Device, CAMAG) and subsequent heating at 120 °C on a TLC plate heater (CAMAG). The intensity of the dyed spots were measured with a CAMAG TLC Scanner 4 at 650 nm wavelength, a slit dimension of 3.00 x 0.30 mm and a data resolution of 100 µm per step. All data were analyzed via winCATS software (CAMAG).

3.2.2.6 Upscaling of the arabinose laurate synthesis

To gain higher amounts of arabinose laurate for CMC value measurements, an upscaling of the synthesis reaction was necessary. To do this, 30.69 g of CC, 33 g of arabinose and 3.18 mL of water were filled in a 250 mL round flask to form a DES in a molar ratio of 1 : 1 and a water content of 5 %. The round flask was heated in a rotatory evaporator at 70 °C until a homogeneous liquid obtained. Subsequently, 1 g of iCalB and 8 mL vinyl laurate (31 mmol) were added. The reaction ran for 48 h at 50 rpm.

To extract the formed arabinose laurate, 50 mL water were added to dissolve the DES. The solution was filtered and residual solids were washed three times with 10 mL of water and three times with 10 mL ethyl acetate. The aqueous phase was extracted six times with 125 mL ethyl acetate in a separator funnel.

Afterwards, the combined organic phase was dried over $MgSO_4$, filtered and concentrated with a rotatory evaporator to obtain an average volume of 10 mL of homogenous liquid.

To gain pure arabinose laurate, the solution was purified in two batches by flash chromatography (Revelersi Prep, Büchi Labortechnik GmbH, Germany) with a 12 g silica column and a gradient of chloroform : methanol with a flow rate of 30 mL/min. The gradient set-up was as follows: 0 % methanol for 1.5 min, 7 % for 9.5 min, 15 % for 3 min and 100 % methanol for 3 min. Product peaks were detected by an evaporative light scattering detector (Treshold: 20 mV, Sensitivity: low), fractionated and analyzed by TLC.

3.2.2.7 Determination of arabinose laurates surface properties

Surface active properties of purified arabinose laurate where determined with a Wilhelmy Plate Tensiometer (DCAT25, DataPhysics Instruments GmbH, Germany; Platin rectangle, 19 mm x 0.2 mm). Arabinose laurate was diluted in pure demineralized H_2O to a stock solution of 10 mM. Afterwards, 12 dilutions between 7.5 mM and 0.03 mM were made and the surface tension of all solutions were measured for 15 min. All measurements were done as single experiments.

3.3 Results and Discussion

3.3.1 Water activity of the DES

The water activity of all 15 used DES was determined. The water activity of the three pure DES is near the measuring limit at around 0.05 (Figure 3.2). The addition of water leads to an increase of the water activity of the DES up to an a_w of 0.15 at a CC:Ara ratio of 2:1, respectively 0.28 at a ratio of 1:2. Besides the amount of water, the water activity depends on the ratio of CC and Ara. The more CC is part of the DES, the lower is the water activity. A reason for this could be the strong hydroscopic effect of CC, which pulls water and binds it tightly.

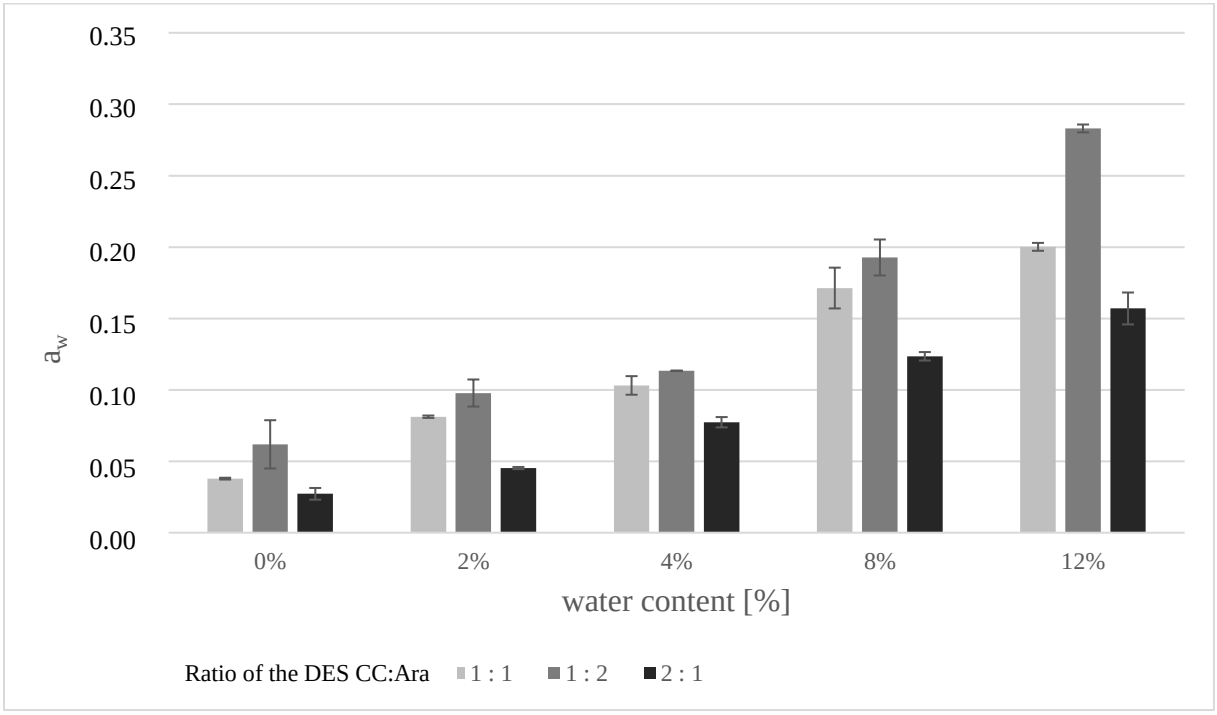

Figure 3.2: Water activity of the DES as a function of the water content. Measurements were done as duplicate.

3.3.2 Time-depending formation of arabinose laurate and di-laurate

The iCalB catalyzed time-depending formation of arabinose laurate and di-laurate in various DES was determined via HPTLC in order to know, under which reaction conditions and time-points synthesis is leading to the highest product yields.

In a DES, based on CC and arabinose in a molar ratio of 2:1 and five different water contents, the time-dependent formation of arabinose laurate varies a lot (Figure 3.3). Per 2 g of DES and 130 µL vinyl laurate, 69945 µg arabinose laurate was formed with 12 % and 69618 µg with 8 % of water in the DES. Using both conditions, a plateau phase was reached after 24 h. By adding 4 %, 8 % or 12 % of water, the formation of arabinose laurate is nearly three times higher than in the pure DES after 48 h, where only 24915 µg arabinose laurate was synthesized.

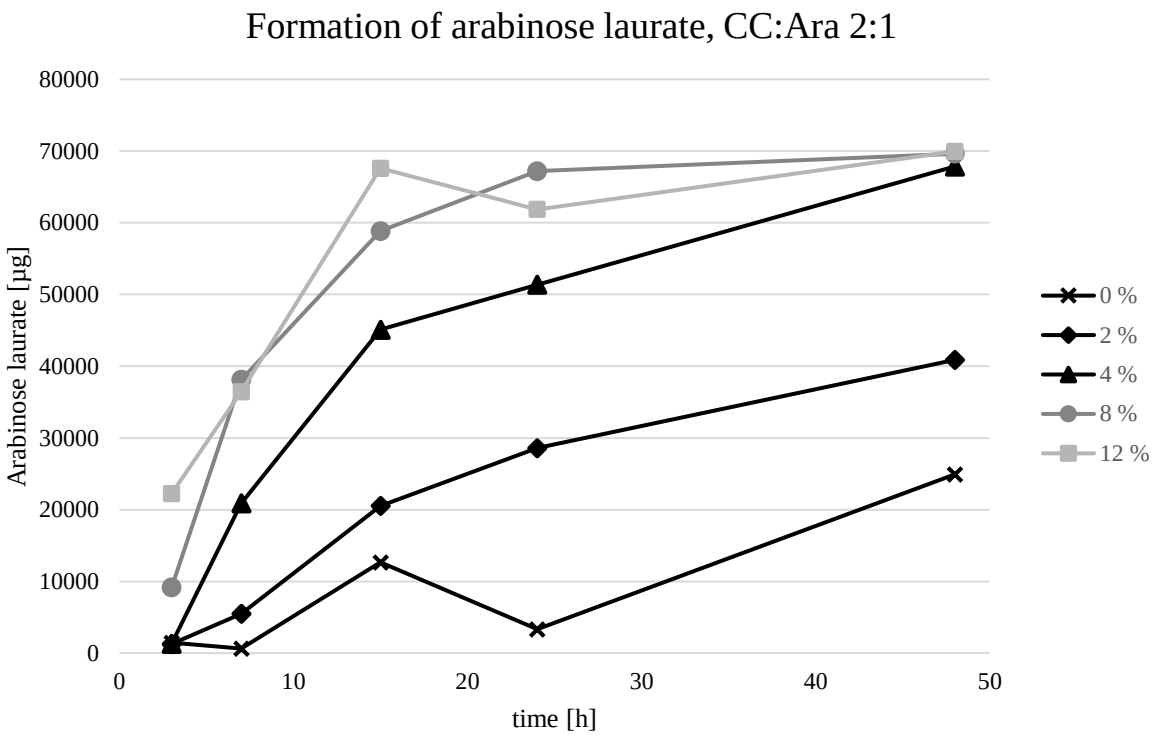

Figure 3.3: Time-depending formation of arabinose laurate in a DES based on CC and Ara with a molar ratio of 2:1 and five different amounts of water.

In the DES with a unimolar ratio of CC and arabinose, the difference between the highest amount of formed arabinose laurate (4 % water, 82391 µg) to the lowest amount (0 % water; 44785 µg) is smaller than in the DES with a molar ratio of 2:1 (Figure 3.4). By using this ratio, it could not be clarified if a plateau was reached, so it has to be further investigated if after more than 48 h more arabinose laurate is formed.

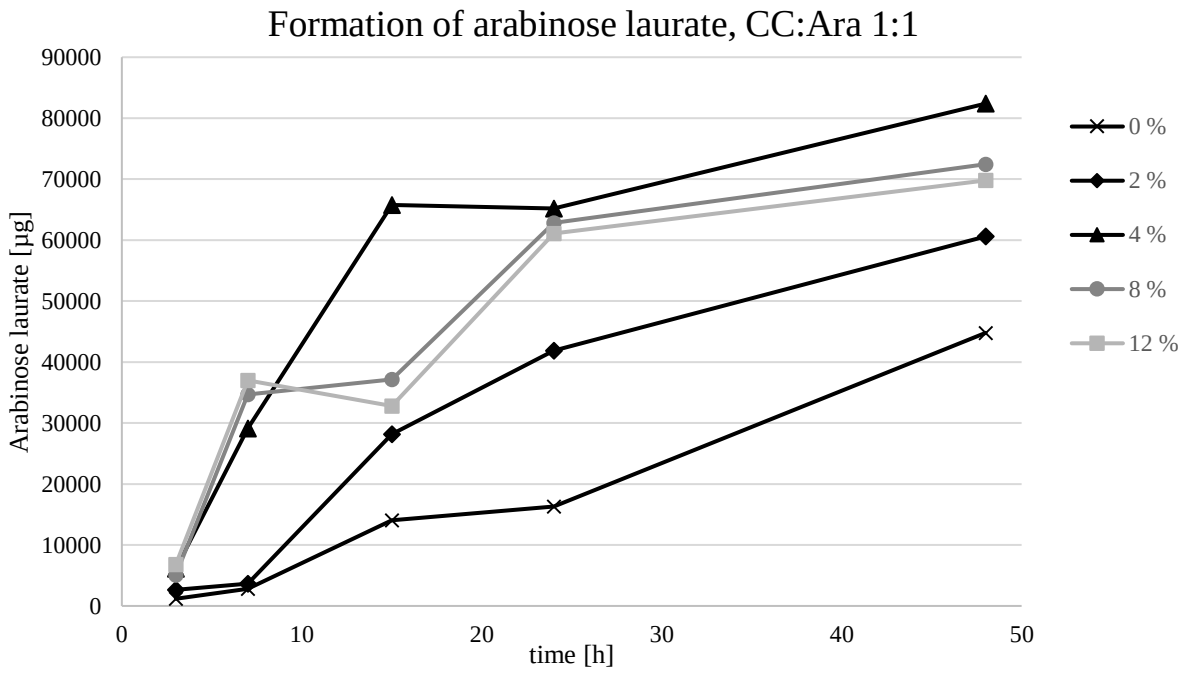

Figure 3.4 Time-depending formation of arabinose laurate in a DES based on CC and Ara with a molar ratio of 1:1 and five different amounts of water.

By increasing the sugar amount in CC:Ara to a ratio of 1:2, the iCalB shows a higher activity and forms in all five DES more arabinose laurate. After 24 h, all reactions with water amounts between 2 % and 12 % reached a plateau with around 75000 µg of formed arabinose laurate (Figure 3.5). Afterwards, there is only a slight increase. In a water-free synthesis reaction, 55130 µg was formed after 48 h, which is the highest amount for water-free reactions.

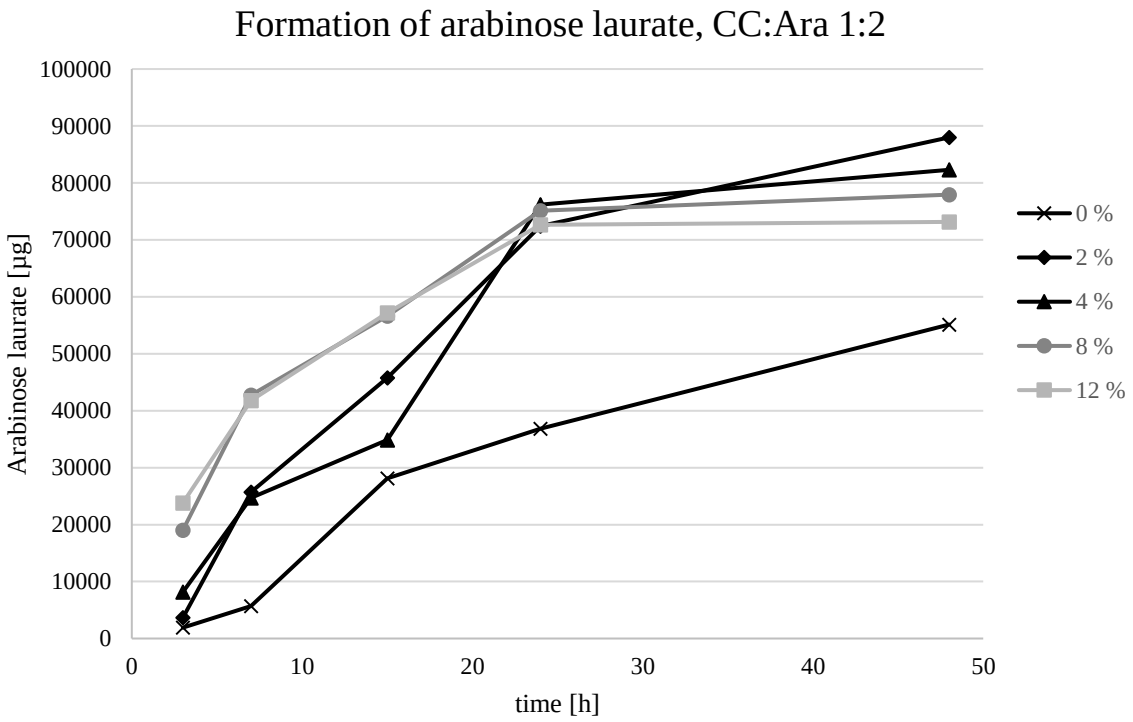

Figure 3.5: Time-depending formation of arabinose laurate in a DES based on CC and Ara with a molar ratio of 1:2 and five different amounts of water.

Further, the yields of the synthesis reaction after 24 h and 48 h were calculated (Table 3.1). To do this, it was necessary to determine how much arabinose laurate could be synthesized. The limiting factor for the maximum yield is the amount of the used vinyl laurate, of which 0.5 mmol was used per synthesis reaction. The other substrate, arabinose, is present in excess (3.88 mmol to 7.6 mmol, depending on the sugar ratio). It can be concluded from the data that only 0.5 mmol of arabinose laurate can be synthesized if there are no other side-products.

Table 3.1: Yields of arabinose laurate in percent of all synthesis reactions after 24 h and 48 h.

time [h] / water cont.	**CC:Ara [2:1]**				
	0 %	**2 %**	**4 %**	**8 %**	**12 %**
24 h	2.0	17.2	31.0	40.5	37.3
48 h	15.0	24.6	40.8	41.9	42.1
time [h] / water cont.	**CC:Ara [1:1]**				
	0 %	**2 %**	**4 %**	**8 %**	**12 %**
24 h	9.8	25.2	39.3	37.8	36.8
48 h	27.0	36.5	49.6	43.6	42.0
time [h] / water cont.	**CC:Ara [1:2]**				
	0 %	**2 %**	**4 %**	**8 %**	**12 %**
24 h	22.2	43.6	45.9	45.3	43.8
48 h	33.2	53.0	49.6	47.0	44.1

The resulting data indicate that the water content, respectively the water activity of the DES has a significant influence on the enzyme activity and product formation. In all reactions with pure DES, the formation of arabinose laurate is much slower and the synthesized amount after 48 h is clearly lower in comparison to all other set-ups. By adding a few percent of water the water activity rises up to an a_w of 0.28 and the yields increased from 15 to 33 % up to 40 to 53 % after 48 h. The effect of an increasing yield and a fastening of the product formation through the addition of water to the DES were observed in other lipase catalyzed reactions carried out in a DES before (Erwann Durand et al., 2013; Guajardo et al., 2017a). The weak lipase activity in pure DES could be explained by strong associations of the arabinose molecules in the DES network, why the sugar is not available for the enzymatic reaction (D'Agostino et al., 2015). Further, the viscosity in pure sugar-based DES is very high in comparison to other solvents and DES (A. Hayyan et al., 2013), the mass transfer could be limited. The addition of water can help to provide sugar to the reaction and to lower the viscosity (Zhang et al., 2012). Further, the water could support the enzymes hydration shell and therefore increase and maintain the catalytic activity (Hari Krishna and Karanth, 2002; Iyer and Ananthanarayan, 2008). However, Chamouleau reported, that with a lower water activity the yields of a lipase catalyzed synthesis reaction in 2m2b will increase (Chamouleau et al., 2001). Whether these results can be transferred to the reaction medium used here must be investigated further.

Despite the large number of measurements, it could not be conclusively clarified which quantity of water in the DES is leading to the best possible yield, because yields vary in all three DES between 2 % (at a ratio of CC:Ara 1:2) and 12 % (at a ratio of 2:1). It can be stated, that a too high water content can lead to a decrease of the yields, which was shown in the DES with a ratio of 1:1 and 1:2 at water contents of 8 % or above. A possible reason could be, that the high hydration level of the DES is increasing the splitting of the product in free lauric acid and sugar (Durand et al., 2014). Further, high amounts of water can destroy the DES hydrogen bond network through unfavorable water clusters, which is leading to a point where the system is best described as an aqueous solution of the DES components (Hammond et al., 2017) which might also have a negative effect on the enzyme. With a DES water content of 4 % it might be possible to increase the reaction yields drastically, while avoiding that water can reduce the formation of arabinose laurate.

The time depending yields show, that in most set-ups the majority of glycolipids will be formed within the first 24 h and that afterwards often a plateau is reached. This could be an important point for an industrial production of the surfactant to optimize the time-yields. Since most reactions reached this product plateau it has to be investigated, whether the enzyme gets inactivated when a thermodynamic equilibrium is reached or if higher fatty acid concentrations are needed.

By comparing the formation of arabinose laurate in all three DES it becomes clear, that more arabinose laurate was formed in the DES with the highest amount of sugar. This could explain, that the sugar is better accessible when the DES contains more sugar.

Minor fluctuations of the amount of formed arabinose laurate on the course of time can be neglected, as the trend is clearly visible. These small errors can be caused by many reasons, such as inaccuracies during the extraction or time intervals. Furthermore, the usage of different iCalB lots can also cause differences in the enzyme activity.

The iCalB catalyzed synthesis of arabinose di-laurate shows a totally different pattern: The formation of di-laurates is strongly controlled of the DES water content. Only in pure DES it is possible to form arabinose di-laurates with higher yields (up to 32 % after 24 h). Even with the addition of 2 % of water, in all other DES less than 5 % of the theoretical yield were formed. This indicates, that the occurrence of water reduces the formation of di-laurates or that formed di-laurate will be cleaved back into the mono-laurate.

To support some of the mentioned assumptions, the viscosity of all DES should be determined for a better understanding of the reaction and media. Therefore, a simulation of the DES nanoscale network and a design of experiments approach could lead to higher reaction yields. After optimizing the media and time, further parameter like temperature or pH should be improved.

3.3.3 Surface active properties of arabinose laurate

Several surface properties were successfully determined using tensiometer measurements. First, it was determined that purified arabinose laurate is able to reduce the surface tension of pure water to air from 72.7 mN/m to 23 mN/m (Figure 3.6). Compared to microbial glycolipids, arabinose laurate shows the highest reduction of the surface tension of demineralized water (Table 3.2). Vice versa, the CMC value of 530 mg/L (1.6 mM) is much higher than of other microbial glycolipids. The strong reduction of surface tension could make the synthesized arabinose laurate to a very promising surfactant for industry, especially for water based surfactant applications due to its relatively short acyl chain, which gives the molecule a more hydrophobic character. Furthermore, the surface excess concentration, which indicates the area-related concentration of a surfactant at the surface interface, is 0.0156 mmol/m^2.

Table 3.2: Comparison of different microbial glycolipids in relation to the surface tension reduction of water and their CMC value.

	Surface tension [mN/m]	CMC [mg/L]	Reference
Arabinose laurate	23	530	This work
Trehalose lipids	25	100	(Vollbrecht et al., 1998)
Rhamnolipids	25 – 30	10 – 200	(Syldatk et al., 1985)
Sophorolipids	30 – 37	82 – 200	(Cooper and Paddock, 1984)
MEL A	28	0.0027 mM	(Kitamoto et al., 1993)
MEL B	28	0.0045 mM	(Kitamoto et al., 1993)

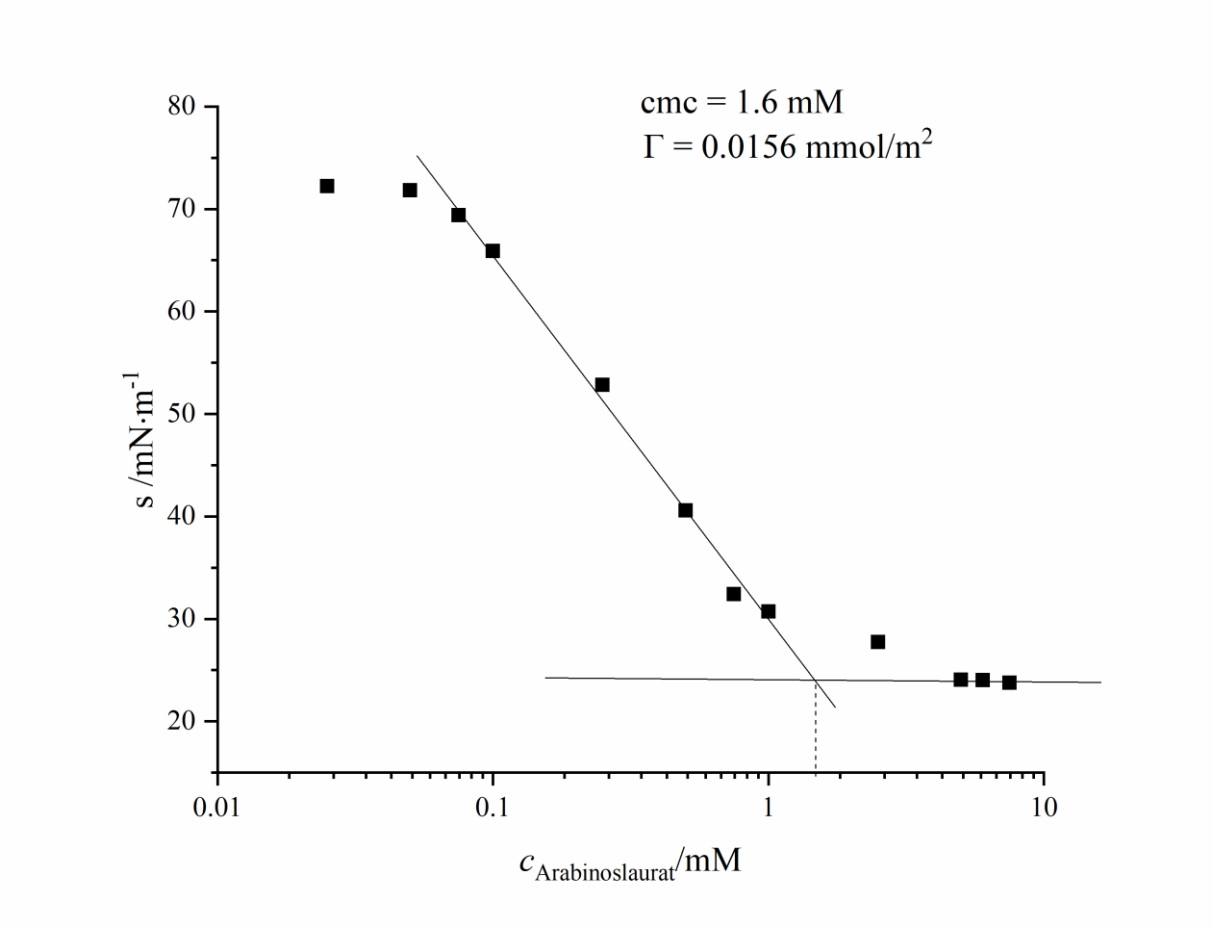

Figure 3.6: Concentration-dependent surface tension values of arabinose laurate in water against air.

Due to low yields and difficulties during purification and measuring, it was not possible to determine the surface activity of arabinose di-laurate.

3.4 Conclusions

The presented study shows that the formation and yield of the lipase catalyzed synthesis reaction of arabinose laurate and di-laurate in arabinose based DES is strongly dependent on the occurrence of water. The addition of 2 % to 12 % of water can more than double the yield to approximately 50 % after 48 h. The reasons for this could be a lower viscosity of the DES and therefore a better mass transfer or an improved availability of the sugar. Despite this, the addition of 12 % water could also lower the formation of arabinose laurate by a few percent in comparison to DES containing only 4 % of water due to a hydrolytic cleavage of the molecule. The formation of di-laurates is almost exclusively possible in anhydrous environments. Moreover, it is possible to control the product formation by the addition of water and lead to a desired direction.

Arabinose laurate shows very good surface tension reducing properties, even in comparison to known glycolipids, and lower the surface tension of water to air to 23 mN/m. The CMC value with 530 mg/L is quite high, but it could be an interesting surfactant for water based application due to its relatively short acyl chain.

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4 Beech wood carbohydrates as resource for sustainable glycolipids

This chapter is based on the following publication

Beechwood Carbohydrates for Enzymatic Synthesis of Sustainable Glycolipids

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Bioresources and Bioprocessing

Volume 4: 25

Available online 7 June 2017

DOI: 10.1186/s40643-017-0155-7

Authors' contribution to this publication

Sascha Siebenhaller planned the study, conducted experiments, analyzed the results and wrote the manuscript. He supervised Tatjana Hajek.

Tatjana Hajek carried out experiments like synthesis reactions, pretreatment of the monosaccharide solution, purification and characterization of formed glycolipids.

Claudia Muhle-Goll, **Miriam Himmelsbach** and **Burkhard Luy** performed the NMR measurements and help to analyze them. Write and proofread parts of the NMR sections.

Frank Kirschhöfer and **Gerald Brenner-Weiß** helped to perform the ESI-ToF experiments. Write and proofread parts of the ESI-ToF sections.

Thomas Hahn and **Susanne Zibek** prepared and analyzed the monosaccharide solution. Proofread the manuscript.

Christoph Syldatk supervised the project and provided scientific input for this study.

4.1 Abstract

Currently, the world's economy is running on crude oil, a limited resource. It is the starting product for fuels, plastics, chemicals and pharmaceuticals; furthermore, it covers a large part of the world's energy supply. But the wastefulness of crude oil causes harm to the earth and the fear about the end of cheap oil is a widespread concern in people's mind.

Therefore, the usage of renewable resources for the production of sustainable compounds is a challenge for future generations. To overcome this, the use of lignocellulose, the most abundant renewable resource of the world, as substrate can contribute to drastically reduce the consumption of crude oil. Lignocellulose is mainly consisting of different carbohydrates, which have to be pretreated first to make theses sugars available for further processes. In this current proof of principle, sugars from lignocellulose were used as starting material for the enzymatic synthesis of surface active sugar esters. The carbohydrates were obtained by an acid-catalyzed, beech wood pretreatment process, which results in a fiber fraction that is subsequently hydrolyzed to obtain the monosaccharides. After purification and drying, this glucose- and xylose-rich fraction was used to create a deep eutectic solvent. In this already established reaction system, the sugars act both as solvent and substrate for the lipase-catalyzed reaction at the same time. Finally, the successful synthesis of glycolipids with a polar head made of a sustainable resource were confirmed by ESI-Q-ToF mass spectrometry and multidimensional NMR experiments. Moreover, conversion yields of 4.8 % were determined by LC-MS/MS.

4.2 Materials and Methods

4.2.1 Materials

Lipase B from *Candida antarctica*, immobilized on acrylic resin (iCalB) and choline chloride (98 %) were purchased from Sigma Aldrich (Sigma Aldrich, St. Louis, USA). $CDCl_3$ (99.8 %) and acetone-d6 (> 99.9 %) were from Eurisotop (Gif sur Yvette Cedex, France). Activated carbon was acquired from Carl Roth (Carl Roth KG, Karlsruhe, Germany). Fatty acids were purchased from Tokyo Chemical Industry Co. Ltd (TCI-Europe, Zwijndrecht, Belgium). All solvents were purchased in the highest purity available and used without further treatment.

4.2.2 Methods

4.2.2.1 Preparation of the monosaccharide solution

The monosaccharide solution applied in the investigations was obtained by a beech wood fractionation via acid-catalysed organosolv process (Figure 4.1). Enzymatic hydrolysis of the fiber and the evaporation of water resulted in the following mono- and disaccharide concentrations in the hydrolysate: 31 g/L cellobiose, 90 g/L xylose and 608 g/L glucose. The samples were diluted to obtain suitable concentrations (< 10 g/L) for HPLC measurement (Ludwig et al., 2013). The chromatographic analysis was performed using an Aminex HPX-87H column (Bio-Rad Laboratories, Germany) as stationary phase and 5 mM sulfuric acid as mobile phase. Separation at 30 °C and a volumetric flow of 0.6 mL/min enabled the identification of single compounds with a refractive index detector (8120, Bischoff Chromatography, Germany). Common degradation products as HMF, furfural or acetic acids which can also be quantified by the application of the HPLC method were either not detected or below the limit of determination. Calibration with pure standards ranged from 0.5 to 10 g/L.

Figure 4.1: Barrel with the used monosaccharide solution which was made via an acid-catalyzed organosolv process from beech wood.

4.2.2.2 Pretreatment of the monosaccharide solution

The monosaccharide solution was further purified to remove other interfering substances like phenolic or lignin compounds which are not detectable with the HPLC method described before (Figure 4.2). For this, the fraction was diluted in ddH_2O (1:2 w/v) and 1 g activated carbon per 5 mL solution was added. After rigorous shaking for 1 min, the activated carbon - sugar mixture was incubated for 3 h at room temperature. Succeeding purification, the activated carbon was separated by a two-step filtration process

(pore size 4-7 µm and 0.22 µm). Afterwards, the sugar solution was spray dried in a werco® SD-20 spray dryer (input temperature 182 °C, output temperature 74 ° - 81 °C; Hans G. Werner Industrietechnik, Reutlingen, Germany). The sugar-mix had a final water content of 8 % (TitroLine® 7500 KF trace, SI Analytics, Mainz, Germany).

Figure 4.2: Scheme of the monosaccharide solution purification. From left to right: Monosaccharide solution diluted in water → solution after incubating with activated carbon and the two-step filtration → during the spray drying → clean and dry sugar-mix.

The two main carbohydrates glucose and xylose were quantified after drying by HPLC (Agilent 1100 Series, Agilent Technology, Waldbronn, Germany) as described in Buchholz *et al.*, 2013 with slight modifications. The flow rate was set to 0.5 mL/min and the temperature of the refractive index detector (Agilent 1200 Series) was set to 50 °C, too.

4.2.2.3 Preparation of deep eutectic solvents

DES for the synthesis of glycolipids from beech wood carbohydrates were prepared by mixing choline chloride with the purified and dried sugar-mix at a given ratio of 1:1.3 (w/w). For the production of glucose- and xylose octanoates as standards, CC and the corresponding sugar were mixed in a molar ratio of 1:1. The mixtures were constantly stirred at 100 °C until a liquid DES was formed.

4.2.2.4 Synthesis and extraction of glycolipids

The synthesis reactions were performed by adding 100 mg of iCalB and 1.5 mmol of the fatty acid (vinyl octanoate or octanoic acid) to 3.5 mL of the formed DES in a 5 mL Eppendorf cup. The synthesis reaction was incubated for 72 h at 50 °C in a rotator with vortex mixer in program U2 at 50 rpm (neoLab, Heidelberg, Germany). Negative controls were carried out without lipase or fatty acid.

After 72 h the DES was dissolved while shaking by adding 1 mL of 70 °C hot ddH_2O until full dissolution of the DES was reached. To perform glycolipid extraction, the solved DES solution was added in a

ratio of 1 : 1 (by vol.) to ethyl acetate and the mixture was shaken for 45 s. Afterwards, the glycolipid containing organic phase was analyzed via thin-layer chromatography.

In order to determine the yields of the enzymatic reaction, it is essential to extract all synthesized glycolipids from the DES. To accomplish that, the solved DES solution was extracted 5 times with ethyl acetate (1 : 1, by vol.). These extracts were merged and used for the LC-MS/MS analysis.

4.2.2.5 Qualitative detection of glycolipids via thin-layer chromatography

For qualitative analysis of glycolipids, 10 µL of extracted samples were spotted onto a silica gel TLC plate (Alugram SIL G, 60 Å, Macherey-Nagel GmbH & Co. KG, Düren, Germany). The separation of synthesized compounds were performed with a mobile phase consisting of chloroform:methanol:acetic acid (65:15:2 or 35:1:1, by vol.). Visualization was accomplished by incubation of the TLC plate in a staining solution (anise aldehyde:sulfuric acid:acetic acid 0.5:1:100, by vol.) followed by heating under a 200 °C continuous air flow for up to 5 min.

4.2.2.6 Purification and fractionation of glycolipid extracts by flash chromatography

The glycolipid extracts were purified by flash chromatography previous to further analysis with mass spectrometry or NMR. For this purpose, multiple extracts from 12 identical synthesis reactions were combined and concentrated to 4 mL (50 mbar, 50 °C and 2.000 rpm; Heidolph Laborota 4000, Schwabach, Germany). The compounds of the concentrated solution were separated by employing a hydrophobic silica column with a Sepacore X50 (40 – 63 µm particle size, 60 Å pore size, 150 mm column length, 12 mm column diameter; Büchi Labortechnik GmbH, Flawil, Switzerland). A gradient of chloroform:hexane (4:1, by vol.) and methanol were used as solvent at a constant flow rate of 5 mL/min. Elutes were collected in 5 mL fractions and analyzed via TLC.

The synthesized glucose- and xylose octanoates to be used as LC-MS/MS standards were purified applying a Reveleris® Prep System with a Reveleris® Silca 12 g column (Büchi Labortechnik GmbH, Flawil, Switzerland). A gradient of chloroform (A) and methanol (B) was used (in 2 min from 0 % to 5 % B, increase the gradient gradually in 10 min to 10 %, then in 1 min to 100 % B and keep it constant for 2 min). Peaks were detected by an evaporating light scattering detector (Threshold 25 mV). The glucose octanoate respectively xylose octanoate fractions were collected, concentrated and transferred to pre-weighed round bottom flasks. The eluent was completely evaporated in a vacuum concentrator (50 mbar, 50 °C and 2.000 rpm; Heidolph Laborota 4000, Schwabach, Germany). The flasks were weighed again to determine the amount of pure sugar esters to be used as standards during LC-MS/MS.

4.2.2.7 Determination of the accurate masses and structure via electrospray ionization quadrupole time of flight mass spectrometry (ESI-Q-ToF MS)

An ESI-Q-ToF MS system (Q-Star Pulsar i, AB SCIEX, Darmstadt, Germany) equipped with an electrospray ionization (ESI) source was used for mass determination of the purified compounds. Measurements were carried out in the positive mode within a mass range from *m*/*z* 50 to *m*/*z* 800 using the activated "enhance all" setting. Samples were diluted 1:5 in a mixture of methanol and 10 mM ammonium acetate (1:1, by vol.) and continuously infused via a syringe pump at a flow rate of 10 µL/min.

The ion source voltage was set at 4,800 V, declustering potential was adjusted at 30 V and focusing potential at 100 V. In all experiments nitrogen gas 5.0 was used as nebulizer and curtain gas.

Data acquisition and processing were performed using the Analyst QS 1.1 software (AB SCIEX, Darmstadt, Germany).

4.2.2.8 LC-MS/MS analyses for glycolipid quantification

Mass spectrometric analyses were done by using an API 4000™ quadrupole mass spectrometer (Applied Biosystems / MDS Sciex Toronto, Canada) equipped with an electro spray ionization (ESI) source. MS spectra were generated by infusion experiments using a syringe pump (Harvard Apparatus Inc. South Natick, MA, USA). Single MS experiments (Q1 scan) and MS/MS experiments (product ion scan, PIS) were used to get structural information. Nitrogen 5.0 was used as curtain gas, nebulizer gas and collision gas.

The purified standard compounds glucose octanoate and xylose octanoate were diluted in a solvent mixture of acetonitrile and 10 mM ammonium acetate (50:50, v/v) and infused with a flow rate of 0.80 mL/h. MS experiments were carried out in the positive ionization mode using an ion spray voltage of 4,800 V, a declustering potential of 30 V and an entrance potential of 10 V. MS/MS experiments were generated using the compound optimization mode in the software Analyst V 1.6.

For both targets three mass transitions (one quantifier and two qualifiers) were selected (Table 4).

Table 4.1: Targets of the LC MS/MS analyses with the corresponding mass transition of the quantifier and both qualifiers.

Target	*m/z* Quantifier	*m/z* Qualifier 1	*m/z* Qualifier 2
Glucose octanoate	307/289	307/271	307/127
Xylose octanoate	277/259	277/127	277/115

A calibration function from 100 to 1,000 µg/L for each sugar ester was prepared for quantification of the extracted targets.

An Agilent 1100 HPLC system (Agilent Waldbronn, Germany) was used for sample separation on a Multospher 120 AQ RP C-18, 5 µm column (250x4 mm). A gradient of acetonitrile (A) and 10 mM ammonium acetate (B) was used by a total run time of 25 min (start with 2 min of 30 % A, increase it in 3 min to 60 % and hold it for 7 min. Afterwards switch in 1 min back to 30 % and hold it for 12 min).

The ion source temperature was set to 400 °C and a flow rate of 500 µL/min was applied. The injection volume for all samples was 40 µL.

4.2.2.9 Structural elucidation via nuclear magnetic resonance spectroscopy

For NMR spectroscopy, 3.9 mg of purified fractions 105-106 (with octanoic acid) from the flash chromatography was dissolved in 0.6 mL $CDCl_3$ and d6-acetone (4:1, by vol.). 1D ^{1}H NMR spectroscopy and 2D ^{1}H-^{1}H correlation spectroscopy (COSY), ^{1}H-^{13}C heteronuclear single quantum coherence spectroscopy (clip-HSQC (Enthart et al., 2008)), and heteronuclear multiple bond correlation spectroscopy (HMBC) were recorded on a Bruker AVANCE II+ 600-MHz spectrometer (Bruker AG, Rheinstetten, Germany) equipped with a BBI probe head. Spectra were analyzed with Topspin 3.2 (Bruker AG) and SpinWorks 3.1.8 software (Marat, University of Manitoba). Intensities were measured from a 1D ^{1}H spectrum acquired with a sixteen scan and four dummy scans. Chemical shifts are referenced to the ^{1}H and ^{13}C resonance of tetramethylsilan.

4.3 Results and Discussion

4.3.1 Properties of the DES reaction media

The purified and dried sugar-mix consists of 71.6 % glucose and 16.6 % xylose; cellobiose could not be detected via HPLC. With this data it was calculated that each reaction approach with 3.5 mL DES contains 1.81 g glucose (10.06 mmol) and 0.42 g (2.8 mmol) xylose.

The prepared DES had a final water content of 5.4 % and a pH of 7.7 (SenTix® Mic, Xylem Analytics, Weilheim, Germany), which fits in the optimum pH range for the used iCalB. Hayyan *et al*. investigated the physical properties of different glucose-based DES and concluded that this kind of DES has a nearly neutral pH because of the low acidity of D-glucose, which made them an ideal media for biological and chemical applications (A. Hayyan et al., 2013).

After a reaction time of 72 h, the DES had a pH of about 5.7, whereas the pH control of pure DES and of a DES with 1.5 mmol vinyl octanoate dropped to approx. pH 6.6.

4.3.2 Glycolipid detection via thin-layer chromatography

To confirm the successful enzymatic formation of sugar esters the extracts were analyzed via TLC (Figure 4.3). In negative controls without lipase or fatty acid two slightly spots were visible. These spots are identical with the standards of pure glucose (RF 0.1) and xylose (RF 0.16) which demonstrated that these spots are sugars of the sugar-mix in the used DES.

The visualized extracts of the synthesis reaction with vinyl octanoate as substrate shows, besides the sugar spots, four more spots. These new spots are potential glucose octanoate and xylose octanoate spots with different conformations. Furthermore, the spots with RF 1 indicate the formation of small amounts of highly nonpolar sugar di- or poly-octanoates (Figure 4.4). This leads to the assumption that iCalB is not specific for mono acylated products under the given conditions and can form traces of di- or poly-acylated sugars and not a defined single product. This result agrees with those of earlier investigations (Siebenhaller et al., 2017b).

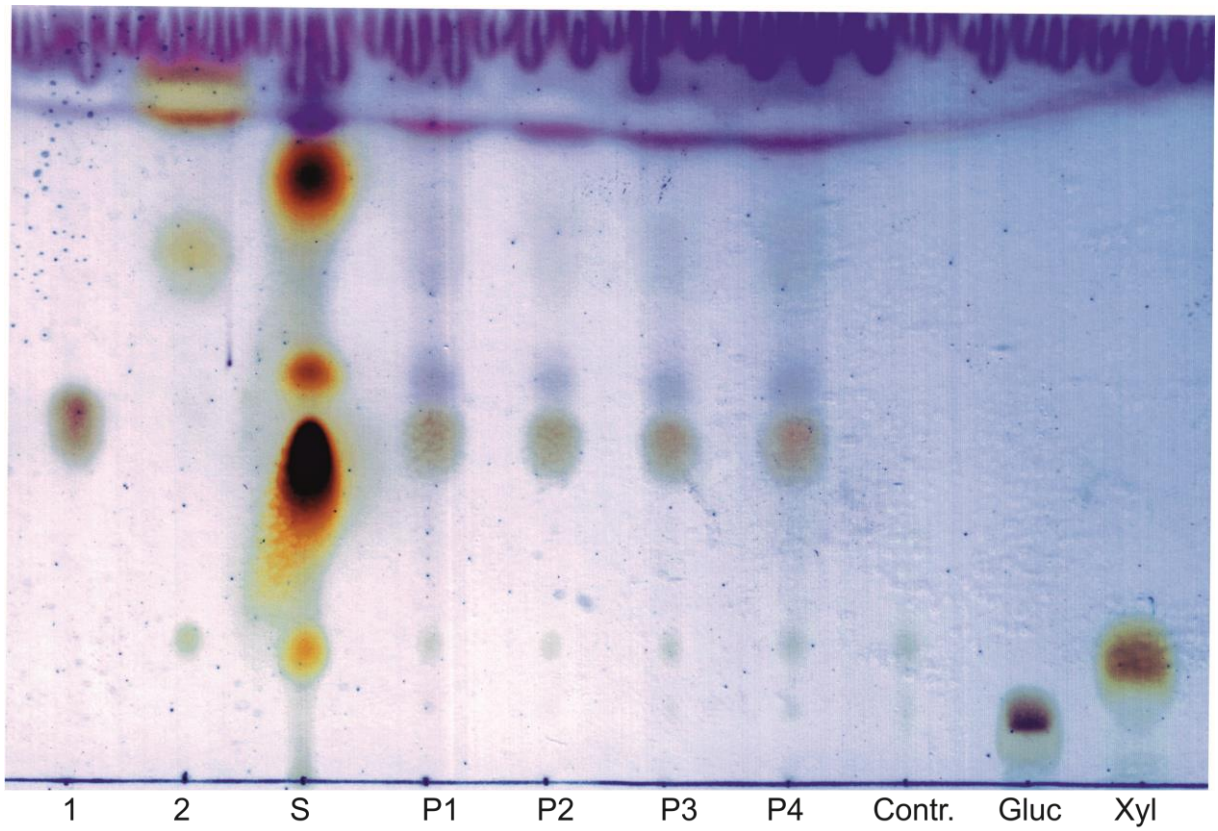

Figure 4.3: The TLC shows the glycolipid standards glucose octanoate (1) and xylose octanoate (2), synthesized with vinyl octanoate. S is a lab intern rhamnolipid standard, which acts as a positive control for the TLC. P1–P4 shows extracts of the reaction in a CC:sugar mix DES with vinyl octanoate as substrate. At a RF between 0.1 and 0.2 there is mainly the sugars, glucose and xylose, out of the DES, which are comparable with the pure sugar standards of glucose (Gluc) and xylose (Xyl). The brownish double spots and the purple spots (P1–P4) represent different glucose octanoates (RF 0.4–0.5) and the slightly spot with a RF of 0.65–0.75 xylose octanoate. The spot on the height of the running front indicates more nonpolar products, like di- or poly-acylated sugars. A negative control without enzyme and without lipase shows only glucose and xylose spots (Contr.)

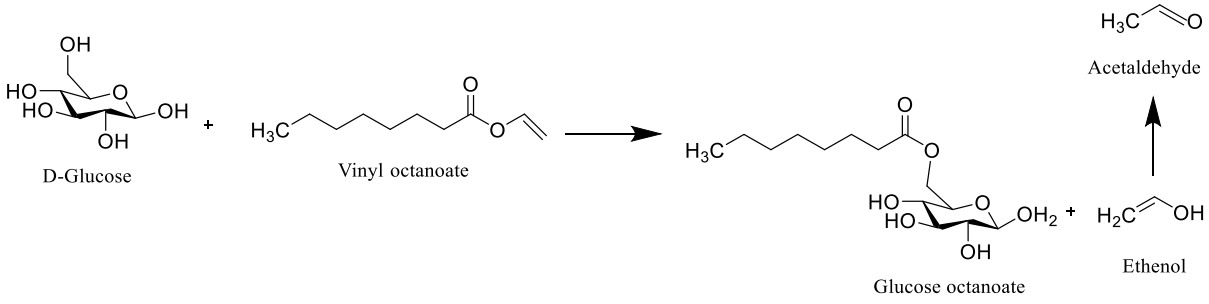

Figure 4.4: Transesterification between glucose and vinyl octanoate which leads to the formation of glucose octanoate and ethenol. Ethenol is not stable and tautomerizes to acetaldehyde. Acetaldehyde evaporates quickly, pushing the reaction forward. The reaction scheme is analogous for other monosaccharides like xylose. It might be possible that more or other C-atoms are acylated, too. Using a fatty acid like octanoic acid, one molecule of water will be formed as a side product.

Moreover, the transesterification with vinyl octanoate additionally releases ethenol, which tautomerizes to a highly volatile acetaldehyde. This should push the reaction forwards and can help to accelerate the reaction (Figure 4.4). On the other hand, under the nearly water-free conditions (approx. 5.4 % water),

it can result in the formation of sugar aldehydes if the acetaldehyde does not evaporate fast enough. To counteract this, a synthesis reaction under reduced pressure might be a possible option.

Further synthesis reactions were made with octanoic acid as substrate, leading to a similar spot pattern (data not shown). By using this substrate, glucose- and xylose octanoate will be formed, too.

Since only qualitative results are obtained by using TLC, samples which possibly contain glycolipids have been characterized further by mass determination and nuclear magnetic resonance spectroscopy.

4.3.3 Mass determination via electrospray ionization quadrupole time-of-flight mass spectrometry (ESI-Q-ToF MS)

For validation of the successful enzymatic synthesis of glycolipids with octanoic acid as substrate, appropriate masses of glucose octanoate (M_G) were detected via ESI-Q-ToF MS in combined fraction 105+106 of the flash chromatography (see Q1 mass spectra in the supplement). The presence of ions at *m*/*z* 271.134 (M_G – 2 H_2O), *m*/*z* 289.148 (M_G – H_2O) and the sodium adduct at *m*/*z* 329.150 (M_G + Na^+) verify the assumed reaction (Figure 4.4).

By using vinyl octanoate as substrate, the masses in combined fraction 26+27 were measured, too. The proof for the correct formation of glucose octanoate is clearly elucidated by the presence of the ion at *m*/*z* 271.150 (M_G – 2 H_2O). Furthermore, the existence of the sodium adduct of glucose di-octanoate (*m*/*z* 455.261) confirms the formation of sugar di-octanoates. All acquired *m*/*z* values of both samples are listed in Table 4.1 and Appendix 9 to Appendix 11.

The formation of glycolipids by iCalB in a DES, consisting of CC and a purified fiber hydrolysate, was thus successful. The proof of the existence of the sodium adducts of glucose octanoate (*m*/*z* 329.150) and glucose di-octanoate (*m*/*z* 455.261) confirms the proposed reaction.

In this two tested fractions no xylose octanoates (M_X) were detected, although xylose is present in small amounts in the fiber hydrolysate, respectively in the sugar-mix.

Even so, the presence of xylose octanoates could be demonstrated with the used substrates in other measured samples. The presence of the sodium adduct of xylose octanoate was identified at *m*/*z* 299.132 in fraction 99 (octanoic acid), in fraction 33-36 the fragment M_X – H_2O was observed at *m*/*z* 259.148 (vinyl octanoate). Since the used pretreated hydrolysate for all DES was the same, xylose octanoates should be formed in low amounts in every reaction.

For complete elucidation of the correct chemical structure of the obtained glucose- and xylose octanoate fraction 105+106 was analyzed using NMR.

Table 4.1: Observed *m/z* values during ESI-Q-ToF experiment of samples 26+27 with vinyl octanoate and 105+106 with octanoic acid are shown. Masses compare to glucose octanoate with a calculated molar mass of 306.168 Da (= M_G) or to glucose di-octanoate of 432.272 Da (= M_{G2}).

Observed *m/z* value	Calculated *m/z* value	Sample	Corresponding fragment
271.134	271.146	105+106 and 26+27	$M_G - 2\ H_2O$
289.148	289.157	105+106	$M_G - H_2O$
329.150	329.157	105+106	$M_G + Na^+$
415.262	415.262	26+27	$M_{G2} - H_2O$
455.264	455.262	26+27	$M_{G2} + Na^+$

4.3.4 Determination of the synthesis yields by LC-MS/MS analysis

Glycolipids from four synthesis reactions with vinyl octanoate, based on a CC:sugar-mix DES, were separately extracted and analyzed by LC-MS/MS. On average, 0.069 mmol of glycolipid (21.4 mg) was formed in one synthesis reaction, of which the main product, with > 97 %, is glucose octanoate (Table 4.2). Only small amounts of xylose octanoate were formed, as a consequence of glucose being four times more abundant than xylose. Furthermore, glucose seems to be favored by the enzyme, which becomes clear when calculating conversion yields for glucose and xylose.

With this set-up, a maximum of 1.5 mmol glycolipids could be synthesized, being this limited by the amount of used octanoic acid. Thus, the overall reaction conversion yield is 4.81 %.

On the other hand, roughly 0.7 % of the glucose in one approach is converted to glucose octanoate, whereas less than 0.1 % of xylose was used to form xylose octanoate.

Synthesized sugar di-octanoates were not included in the calculation of the yield due to their low occurrence.

Although this has been a non-optimized synthesis set-up, it was still possible to produce small amounts of different glycolipids from beech wood carbohydrates. When compared to other enzymatic glycolipid synthesis reactions in organic solvents the achieved yields are quite low. Depending on the solvent, enzyme, substrates and time, as well as the addition of molecular sieves it is possible to convert 100 % of the sugar to a glycolipid (Amos et al., 1998), but in other systems the yield is lower (Ducret et al., 1996; Tarahomjoo and Alemzadeh, 2003).

Table 4.2: Measured amounts of synthesized glucose octanoate and xylose octanoate in one reaction set-up with 3.5 mL of DES, 100 mg iCalB and 1.5 mmol vinyl octanoate. The total amount of approx. 0.07 mmol synthesized glycolipid correspond to a yield of 4.81 %.

Reaction	Yield [%]	Total amount [mg]	Glucose octanoate [mg]	Xylose octanoate [mg]
P1	5.1	22.7	22.1	0.67
P2	4,9	21.7	21.1	0.62
P3	4.6	20.6	20.0	0.64
P4	4.6	20.5	19.9	0.61

To achieve higher yields, the process can be improved by optimizing various parameters. One such parameter is the water content of the DES. It has already been shown that enzymatic reactions can be accelerated by increasing the water content of the DES, so it is important to find the optimal water concentration (Erwann Durand et al., 2013; Guajardo et al., 2017b). Furthermore, it should be possible to set the pH to the lipase optimum by adding a buffer to the DES and not just pure water. Another parameter to improve is the amount of the used enzyme, as well as the fatty acid, since high concentrations can have negative effects on the enzyme. The adjusted temperature of 50 °C is in the optimum range of the lipase, but it is also stable and active at slightly higher temperatures. Increasing the temperature a few degrees would decrease the viscosity (A. Hayyan et al., 2013), which will lead to a better mass transport. Moreover, the mixing of the synthesis reaction has to be improved to create a more homogenous reaction mixture, which will improve the yields.

4.3.5 Configuration of sugar octanoates structure by NMR

The sample 105+106 was analyzed with ^{1}H COSY, ^{13}C HSQC and ^{1}H^{13}C HMBC spectra. Two major carbohydrate systems were identified and studied, starting from the anomeric protons of the ^{1}H COSY and ^{13}C HMBC spectra. Both systems are C6 sugars which were identified as α- and β-glucose. The glucose moieties are acylated with octanoic acid at the C6-atoms, confirmed on the basis of cross peaks of protons with lipid carbonyls in the ^{1}H^{13}C HMBC (Table 4.3).

Furthermore, sample 101+102 were analyzed (supplementary 1). In this sample two major carbohydrate systems were identified. These systems are β-glucoses which were acylated with the fatty acid at the C6, too.

In addition to the main systems there are minor systems present with many overlaps. One of these minor systems could be identified as the pentose β-xylose. This β-xylose is acylated at the OH-group at the C4.

Compared to the two glucose systems, the β-xylose system is acylated on the OH-group at the C4 with octanoic acid. The detection of xylose octanoate in this fraction was expected, based on the results of the ESI-Q-ToF MS measurements.

These NMR-data confirm the results of the previous mass spectrometry. The synthesis of glucose-6-octanoate and xylose-5-octanoate in a DES consisting of CC and sugars from a lignocellulose hydrolysate with iCalB was successfully proven.

From the collected data it can be concluded so far that different sugar molecules may have different preferred acylation sites. In previous work, arabinose, a pentose, was used as sugar for the synthesis of

glycolipids, vinyl laurate served as fatty acid component (Siebenhaller et al., 2017). Two acylated arabinose systems were detected in NMR measurements. One system was acylated at the C3 and the other at C4, or one system was di-acylated at C3 and C4 while the other system was pure arabinose.

The C4 hydroxy group is maybe favored at pentoses like arabinose and xylose, but the other hydroxyl groups are potential acylation targets, too. In glucose, a hexose, the C6 OH-group seems to be favored, because this group has less steric effects due to its position and offers a good access for iCalBs active site.

Table 4.3: Chemical shifts of the main products present in fraction 105+106. d = doublet; t = triplet; n.d. = not determinable.

α-glucose	**C shift [ppm]**	**H shift [ppm]**	**Multiplicity**	**Coupling [Hz]**
─C^1H**─O─**	92.50	5.27	d	2.83
─C^2H**─**	72.45	3.53	t	9.40
─C^3H**─**	73.97	3.82	t	9.40
─C^4H**─**	70.18	3.41	t	9.40
─C^5H**─**	69.56	4.03	d	9.40
─C^6H**─ (*** acylated C`173.93)	63.20	4.30	overlap	n.d.
─$C^{6'}$H**─ (*** acylated C`173.93)	63.20	4.40	overlap	n.d.
β-glucose	**C shift [ppm]**	**H shift [ppm]**	**Multiplicity**	**Coupling [Hz]**
─C^1H**─O─**	96.87	4.64	d	6.40
─C^2H**─**	74.88	3.35	t	8.60
─C^3H**─**	76.37	3.55	t	8.80
─C^4H**─**	70.18	3.46	overlap	n.d.
─C^5H**─**	74.03	3.54	overlap	n.d.
─C^6H**─ (*** acylated C`174.02)	63.40	4.32	overlap	n.d.
─$C^{6'}$H**─ (*** acylated C`174.02)	63.40	4.37	overlap	n.d.

* Acylation site

4.4 Conclusions

Albeit having used a non-optimized process, a first step for the production of sustainable glycolipids from beech wood hydrolysates was achieved. The sugars used for the enzymatic synthesis of sugar esters were extracted from beech wood lignocellulose, a renewable and widely available resource. When forming a DES with these sugars, they are easily available in the reaction solvent. This is an elegant way to avoid the negative effect of the low solubility of sugars in other water-free solvents. The successful formation of different glucose- and xylose octanoates was confirmed by ESI-ToF mass spectrometry and multidimensional NMR measurements. With the used reaction set-up, it was possible to achieve a yield of 4.81 % of the maximum yield, but there is great potential to further optimize the system, by addressing various parameters.

4.5 References chapter 4

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5 Enzymatic production of glycolipids completely based on lignocellulosic substrates

This chapter is based on the publication

Integrated Process for the Enzymatic Production of Fatty Acid Sugar Esters Completely Based on Lignocellulosic Substrates

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Frontiers in Chemistry

Volume 6, Article 421

Published: 13 September 2018

DOI: 10.3389/fchem.2018.00421

Authors' contribution to this manuscript

Sascha Siebenhaller performed experiments, conception of the work, coordination of experiments and supervision of Jennifer Kirchhoff. Writing of the enzymatic and other parts of the manuscript and proof-reading.

Jennifer Kirchhoff performed most experiments (fermentation and synthesis reactions) and revising the final version.

Frank Kirschhöfer and Gerald Brenner-Weiß performed the ESI-Q-ToF measurements, giving intellectual input to the MS section in revising the manuscript.

Claudia Muhle-Goll and Burkhard Luy executed and evaluated the NMR analysis and revising the manuscript.

Fabian Haitz, Thomas Hahn and Susanne Zibek prepared and analyzed the beech wood cellulose fiber hydrolysate. Performed the TLC-MALDI-ToF experiments. Critical proofreading of the manuscript.

Christoph Syldatk critical revision of the work and important intellectual content.

Katrin Ochsenreither coordinated experiments and supervised Jennifer Kirchhoff. Intellectual content and writing the main part of the SCO fermentation and proofread the manuscript.

5.1 Abstract

Lignocellulose can be converted sustainably to fuels, power and value-added chemicals like fatty acid esters due its composition of various carbohydrates. This chapter presents a concept for the first eco-friendly enzymatic synthesis of economically important fatty acid sugar esters based on lignocellulosic biomass. To achieve this, beech wood cellulose fiber hydrolysate was applied in three manners: as sugar component, as part of the deep eutectic solvent (DES) reaction system and as carbon source for the microbial production of the fatty acid component. These fatty acids were gained from single cell oil produced by the oleaginous yeast *Cryptococcus curvatus* cultivated with cellulose fiber hydrolysate as carbon source. Afterwards, an immobilized *Candida antarctica* lipase B was used as the biocatalyst in DES to esterify sugars with fatty acids. Properties of the DES were determined and synthesized sugar mono- and di-esters were identified and characterized using TLC, MS and NMR. Using this approach, sugar esters were successfully synthesized which are 100 % based on lignocellulosic biomass.

5.2 Material and Methods

5.2.1 Preparation of beech wood cellulose fiber hydrolysate

Chopped beech wood was incubated at temperatures >140 °C in aqueous ethanol solution with a small amount of H_2SO_4 as catalyst to obtain different fractions (acid-catalyzed organosolv process). After washing, the fiber fraction, mainly containing the cellulose and a part of hemicellulose, was directly subjected to enzymatic hydrolysis. Enzymatic hydrolysis of the fiber was performed for 24 h at a temperature of 50 °C with a 10 % (w/v) suspension. For stirrer description see Ludwig et al. (Ludwig et al., 2014). pH of the suspension was adjusted to pH 4.8 during hydrolysis using a concentrated NaOH solution. The hydrolysis reaction started by adding 60 mg Cellic® CTec3 and 2.5 mg Cellic® HTec3 per g cellulose. Solid material was removed afterwards applying an extruder press. The successive concentration of the filtrate resulted in the following mono- and disaccharide concentrations: 30.8 g/L cellobiose, 89.7 g/L xylose and 608.3 g/L glucose quantified via chromatographic analysis (see Sluiter et al. (2008) for further description). Analysis furthermore revealed the absence of toxic or degradation products.

The obtained cellulose hydrolysate was directly used as carbon source for SCO production. For the preparation of DES, however, it had to be further purified and dried: The cellulose hydrolysate was diluted in ddH_2O (1:2 w/v) and 1 g activated carbon per 5 mL solution was added. After rigorous shaking for 1 min, the activated carbon mixture was incubated for 3 h at room temperature. Succeeding purification, activated carbon was separated by a two-step filtration process (pore size 4–7 and 0.22 µm). Afterwards, the solution was spray dried in a werco® SD-20 spray dryer (input temperature 182 °C, output temperature 74–81 °C; Hans G. Werner Industrietechnik, Reutlingen, Germany) resulting in a final water content of 8 %, determined by Karl Fischer titration (TitroLine® 7500 KF trace, SI Analytics, Mainz, Germany). Mono- and disaccharide concentrations were analyzed by HPLC.

5.2.2 Production of single cell oil in a bioreactor

5.2.2.1 Strain and medium

For the production of single cell oil, *C. curvatus* ATCC 20509 was used. Cultivation was conducted as outlined by Dillschneider et al. (2014). Briefly, the culture medium was based on a phosphate buffer pH 5 (8.99 g/L KH_2PO_4 and 0.12 g/L $Na_2HPO_4 \cdot 2\ H_2O$). The medium constituents were 0.1 g/L sodium citrate ($C_6H_5O_7Na_3 \cdot 2\ H_2O$), 0.1 g/L yeast extract, 0.2 g/L $MgSO_4 \cdot 7\ H_2O$ and 18.9 g/L $(NH_4)_2SO_4$. Medium was supplemented with 2 mL trace elements solution (4 g/L $CaCl_2 \cdot 2\ H_2O$, 0.55 g/L $FeSO_4 \cdot 7\ H_2O$, 0.475 g/L citric acid, 0.1 g/L $ZnSO_4 \cdot 7\ H_2O$, 0.076 g/L $MnSO_4 \cdot H_2O$, 100 µL18 M H_2SO_4) and 1 mL salt solution (20 g/L $MgSO_4 \cdot 7\ H_2O$, 10 g/L yeast extract) per 100 mL cultivation medium. During cultivation, salts and trace elements were added once every 24 h to the culture broth. Unpurified cellulose fiber hydrolysate was used as carbon source. The hydrolysate was diluted with distilled water to obtain a stock solution with a concentration of 500 g/L carbohydrates. The initial sugar concentration in

the culture medium was 50 g/L. Stock solution was fed daily to keep sugar concentration constant between 50 g/L and 90 g/L during the whole cultivation.

5.2.2.2 Cultivation and Sampling

The first pre-culture was prepared in 20 mL medium in 100 mL conical shake flasks and was inoculated with 100 µL glycerol stock culture of *C. curvatus* (15 % w/w, stored at −80 °C). The second pre-culture with 200 mL culture medium in 2 L shake flasks was inoculated with the first pre-culture to obtain an initial OD_{600} of 1. Both pre-cultures were incubated at 28 °C with 120 rpm for approximately 24 h.

Fermentation of the main-culture was performed in duplicate in a 2.5 L bioreactor (Infors HT, Bottmingen, Switzerland; Minifors fermentor) with 1.2 L culture medium at the beginning, initial OD_{600} of 1, at 600 rpm, 28 °C and with 1 vvm aeration rate for 96 h. Stirrer speed was increased to 800 rpm when dissolved oxygen concentration decreased below 15 %. The pH was adjusted to 5.0 by controlled addition of 4 M H_3PO_4 and 4 M NaOH. Contraspum A 4050 HAC (Zschimmer und Schwarz) was applied as antifoam agent.

Each day four samples were taken as given in Figure 5.2. Prior to sampling, 20 mL of culture broth was discarded. Sampling volume was 5 mL (three samples) or 20 mL (one sample). Samples were subjected to OD_{600}, dry biomass and sugar concentration measurement. Lipid content and composition were only determined in the 20 mL sample, ammonium content only in the first samples until complete consumption. Feeding of salts, trace elements and carbon source was done 30 min before taking the last sample of the day.

5.2.2.3 Single cell oil extraction and transesterification

The biomass of each 20 mL sample and the complete biomass in the bioreactor at the end of the cultivation was collected by centrifugation (5 min, 4,600 x g), washed once in sterile saline and pelleted again by a second centrifugation. Supernatants were discarded and the resulting cell pellet was immediately frozen in liquid nitrogen. Biomass was freeze-dried for at least 24 h, at -30 °C and 0.37 mbar.

For analytical purposes and glycolipid synthesis, fatty acid methyl esters were formed by transesterification from single cell oil as described below. For lipid analysis, 20 mg of freeze-dried biomass was incubated for 2 h at 100 °C in a thermoshaker at 1,400 rpm in the presence of 1.5 mL n-hexane, 2 mL 15 % H_2SO_4 in methanol and 0.5 mL of an internal standard (2 mg/mL methyl benzoate). After cooling on ice, 1 mL demineralized water was added. The mixture was centrifuged for 5 min at 2,500 rpm. 1 µL of the upper phase, containing the fatty acid methyl esters extract, was analyzed via gas chromatography.

For the production of glycolipids, 1 g of freeze-dried biomass was transesterified in the presence of 25 mL n-hexane and 25 mL 15 % H_2SO_4 in methanol for 5 h at 90 °C in an oil bath under constant stirring (350 rpm). After cooling on ice, 5 mL demineralized water was added. The upper organic phase was collected and solvent in this phase was evaporated at 40 °C and 2,000 rpm in a speed vac. The produced fatty acid methyl esters were used as substrate for glycolipid synthesis.

5.2.2.4 Analysis of bioreactor samples

Dry biomass was analyzed gravimetrically. 1 mL of the culture broth was transferred into a pre-dried and pre-weighed reaction tube and centrifuged at 13,000 rpm for 5 min. The supernatant was collected and used for the determination of sugar and ammonia content. The cell pellet was washed with saline, dried at 60 °C for 24 h and weighed.

Ammonium concentration was determined enzymatically using Spectroquant kit (Merck KGaA, Darmstadt, Germany) following the instructions of the manufacturer.

For the carbohydrate quantification with HPLC, fermentation broth samples were pre-treated and analyzed as described by Buchholz et al. (2013) with slight modifications. A protocol for phosphate precipitation was applied before measurement. 1 mL of culture supernatant was mixed with 45 µL 4 M NH_3 and 100 µL 1.2 M $MgSO_4$, incubated for 5 min at RT and subsequently centrifuged for 5 min at 13,000 rpm. 500 µL of the resulting supernatant was then mixed with 500 µL 0.1 M H_2SO_4, incubated for 15 min followed by centrifugation for 15 min at 13,000 rpm. The supernatant was used for HPLC analysis of the carbohydrates as described by Siebenhaller et al. (2018). Calibration with pure glucose and xylose ranged from 10 mg/L to 500 mg/L.

Lipid content and composition were determined by analyzing the fatty acid methyl esters, obtained by transesterification, via GC (Agilent Technologies, 6890 N Network GC-System). The instrument was equipped with a DB Wax column (l: 30 m; d: 0.25 mm, Agilent Technologies Deutschland GmbH, Böblingen, Germany), and a flame ionization detector. The working pressure was 1.083 bar and initial temperature 40 °C. The column temperature was increased from 40 °C to 250 °C with a rate of 8 °C/min. The temperature was maintained at 250 °C for 20 min before cooling down to 40 °C. Fatty acids were identified and the total fatty acid content was determined with the standard FAME Mix RM-3 (Supelco).

5.2.3 Enzymatic synthesis of glycolipids

5.2.3.1 Preparation and evaluation of the deep eutectic solvent

The purified and dried cellulose hydrolysate fraction consists of 71.6 % glucose as well as 16.6 % xylose and was mixed with choline chloride in a ratio of 1:1 (w/w). Afterwards, the mixture was stirred and

heated up to 100 °C until the liquid DES was formed. The DES was directly used as solvent and substrate for the enzymatic glycolipid synthesis.

DES viscosity was determined at 25 °C and 50 °C with a rheometer (5 cm plate diameter, 1 mm gap, shear rate 0-222 [1/s]; Physica MCR 301, Anton Paar GmbH, Austria). The water activity was measured of a fresh DES with a LabMaster-aw neo (Novasina AG, Switzerland).

5.2.3.2 Synthesis reaction and extraction of glycolipids

Glycolipids were synthesized in 5 mL Eppendorf cups by adding 100 mg of iCalB (Lipase acrylic resin from C. antarctica, Sigma-Aldrich, Taufkirchen, Germany) to 2.5 mL of DES. To start the reaction, 175 µL of the prepared fatty acid methyl esters were added and the reaction was performed at 50 °C for 70 h in a rotator with vortex mixer (neoLab, Heidelberg, Germany) in program U2 at 50 rpm. As controls, reactions without enzyme or without fatty acids were performed.

To stop the reaction, 2 mL of warm water was added and the mixture was shaken until full dissolution of the DES. Organic phase extraction with the same volume of ethyl acetate resulted in the accumulation of glycolipids after 45 s of mixing. The glycolipid containing organic phase was removed for further experiments. To enhance extraction efficiency, the addition of ethyl acetate with subsequent mixing can be repeated up to five times.

5.2.4 Purification of glycolipids by flash chromatography

Before further analysis via mass spectrometry and NMR, eight extracts from identical synthesis reactions were unified and purified via flash chromatography (Reveleris Prep, Büchi Labortechnik GmbH, Germany). The extract was mixed with 2 g of silica (40 µm pore size) and liquid was subsequently evaporated in a rotary evaporator. The silica including the bound components (products and excess of sugar and FAMEs) were packed in an empty column. For separation, a Reveleris HP Silica 4g column and a flow rate of 15 mL/min was used. As method, the dry sample function was selected and a gradient of chloroform and methanol was used as follows: 0 % to 7 % methanol in 1 min, holding the gradient for 7 min, followed by an increase to 15 % methanol in 2 min and holding it for 1 min with subsequent increase to 20 % in 1 min. To eluate sugars and other polar components, the gradient was set to 100 % methanol in 1 min holding it for 4 min. Peaks were observed by an evaporative light scattering detector (Threshold: 30 mV, Sensitivity: low) and fractions collected. Unprocessed fractions were analyzed via TLC and divided into three samples (1 = fraction 6-9, 2 = fraction 14+15+17, 3 = fraction 16). For ESI-Q-ToF MS, MALDI-ToF MS and NMR analysis the samples were evaporated.

5.2.5 Analysis of glycolipids

5.2.5.1 Qualitative detection of glycolipids via thin-layer chromatography

As a fast qualitative detection method of the formed glycolipids, 10 µL extracts were spotted onto a 60 Å silica gel TLC plate (Alugram Xtra SIL G, Macherey-Nagel GmbH & Co.KG, Düren, Germany). Compounds were separated using a mobile phase consisting of chloroform : methanol (70 : 5, by vol.). Visualization was accomplished by dipping the TLC plate into an anise aldehyde solution (anise aldehyde : sulfuric acid : acetic acid 0.5 : 1 : 100, by vol.) with subsequent heating under a 200 °C air flow for 5 min.

5.2.5.2 Determination of masses via thin-layer chromatography coupled matrix-assisted laser desorption/ionization time of flight

With the TLC-MALDI-ToF technique it is possible to determine the mass of the product spots on a TLC plate. Two identical replicates of a HPTLC plate (5 x 7.5 cm HPTLC plate, silica gel 60, Merk, Darmstadt, Germany) were thus prepared. 10 µL to 15 µL of the purified and concentrated sample 1 and sample 2 from flash chromatography were sprayed as 6 mm horizontal bands on the TLC plate using an automatic sample application device (ATS4, CAMAG, Switzerland) and were separated with the solvent system chloroform : methanol (70 : 10, by vol.). One plate was dyed with an anise aldehyde solution. On the second plate, a drop of an external polymer standard (Polypropylenglycol M_N 725 (Sigma-Aldrich); 1:100 with methanol) was placed in a corner, afterwards the plate was dipped for 2 s in a matrix solution (200 g/L dihydroxybenzoic acid in 90 % acetonitrile, 1.15 g/L diammonium phosphate, 0.2 g/L octyl β-D-glucopyranoside, 0.1 % trifluoroacetic acid). Subsequently, the plate was fixed onto the MTP TLC Adapter for a Bruker Ultraflex II ToF/ToF (Bruker AG, Rheinstetten, Germany). The dyed plate served as a template providing a coordinate system for the laser pulses (25 kV ion source, offset 63 %, range 15 %, positive mode). Every 0.6 mm of the vertical coordinate system, measurements were made at seven juxtaposed positions. The measured dataset was analyzed by the instrument software flexControl 3.0.

5.2.5.3 Sample preparation and MALDI-ToF mass spectrometry

Sample 1 from flash purification were mixed with MALDI matrix solution (ratio 50 : 50). The matrix solution consisted of 10 mg/mL α-cyano-4-hydroxycinnamic acid (Sigma–Aldrich, Taufkirchen, Germany) in water/aceto¬nitrile (50 : 50, by volume) with 0.2 % (volume ratio) trifluoroacetic acid. 1 µL of the mixture was then spotted on the MALDI target and air-dried.

MALDI-ToF MS experiments were carried out on a 4800 MALDI-ToF mass spectrometer (Applied Biosystems/MDS SCIEX, Foster City, CA) in reflector positive ion mode (focus mass 500 Da). Each

mass spectrum was an average of 1.000 laser shots over the entire spot. Mass calibration was performed using a calibration solution (Sigma-Aldrich) spotted nearby the samples.

5.2.5.4 Verification of the accurate masses via electrospray ionization quadrupole time of flight mass spectrometry

Accurate masses of synthesized and purified glycolipids from sample 2 were determined with an ESI-Q-ToF MS system (Q-Star Pulsar i, AB SCIEX, Darmstadt, Germany) equipped with an electrospray ionization (ESI) source. Measurements were accomplished as described in Siebenhaller et al. (2017). Data acquisition and processing were performed using the Analyst QS 1.1 software (AB SCIEX, Darmstadt, Germany).

5.2.5.5 Structural elucidation via nuclear magnetic resonance spectroscopy

For NMR spectroscopy of formed sugar esters, 30 mg of purified sample 3 from the flash chromatography was dissolved in 0.6 mL CDCl3/d6-acetone (4 : 1, by vol.). 1D 1H NMR spectroscopy and 2D 1H-1H correlation spectroscopy (COSY), 1H-13C heteronuclear single quantum coherence spectroscopy (clip-HSQC (Enthart et al., 2008)), and heteronuclear multiple-bond correlation spectroscopy (HMBC) were performed as described in Siebenhaller et al. (2016).

5.3 Results and Discussion

For the sustainable production of fatty acid sugar ester biosurfactants monosaccharides originated from enzymatically hydrolyzed cellulose fiber fraction and fatty acid methyl esters derived from microbially produced triacylglycerols (single cell oil) are esterified. In a first step, the production of single cell oil based on cellulose fiber fraction and their transesterification to fatty acid methyl ester (FAME) is reported (5.3.1). For the enzymatic esterification the DES reaction system consisting of cellulose fiber monosaccharides and choline chloride was prepared and characterized (5.3.2). Besides as part of the solvent, these monosaccharides act also as substrate for the enzymatic biosurfactant production and are linked to the added FAMEs by iCalB lipase. The successful formation of sugar esters were qualitatively proven by TLC (5.3.3), mass analysis (5.3.4) and NMR (5.3.5). The flowchart of this study is given in Figure 5.1.

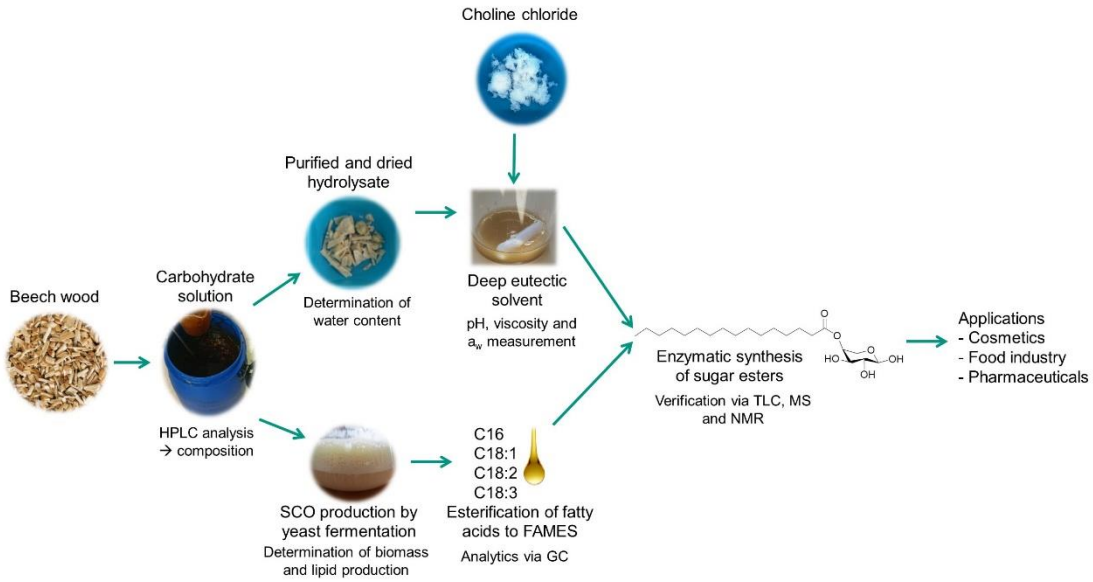

Figure 5.1: Flowchart of the study. Carbohydrates from beech wood cellulose fiber hydrolysates were used to produce fatty acids in a yeast bioprocess, to form the solvent system and as substrate for the enzymatic reaction to produce fatty acid sugar esters (a.k.a glycolipids). Analytical methods are indicated at each work package.

5.3.1 Production of single cell oil using cellulose fiber hydrolysate

The fatty acid component for the synthesis of glycolipids was gained from single cell oil produced by *C. curvatus* with cellulose fiber hydrolysate as substrate. Bioreactor cultivation was conducted in duplicate and the time course is shown in Figure 5.2 as mean of both cultivations. All data are visible in Appendix 12.

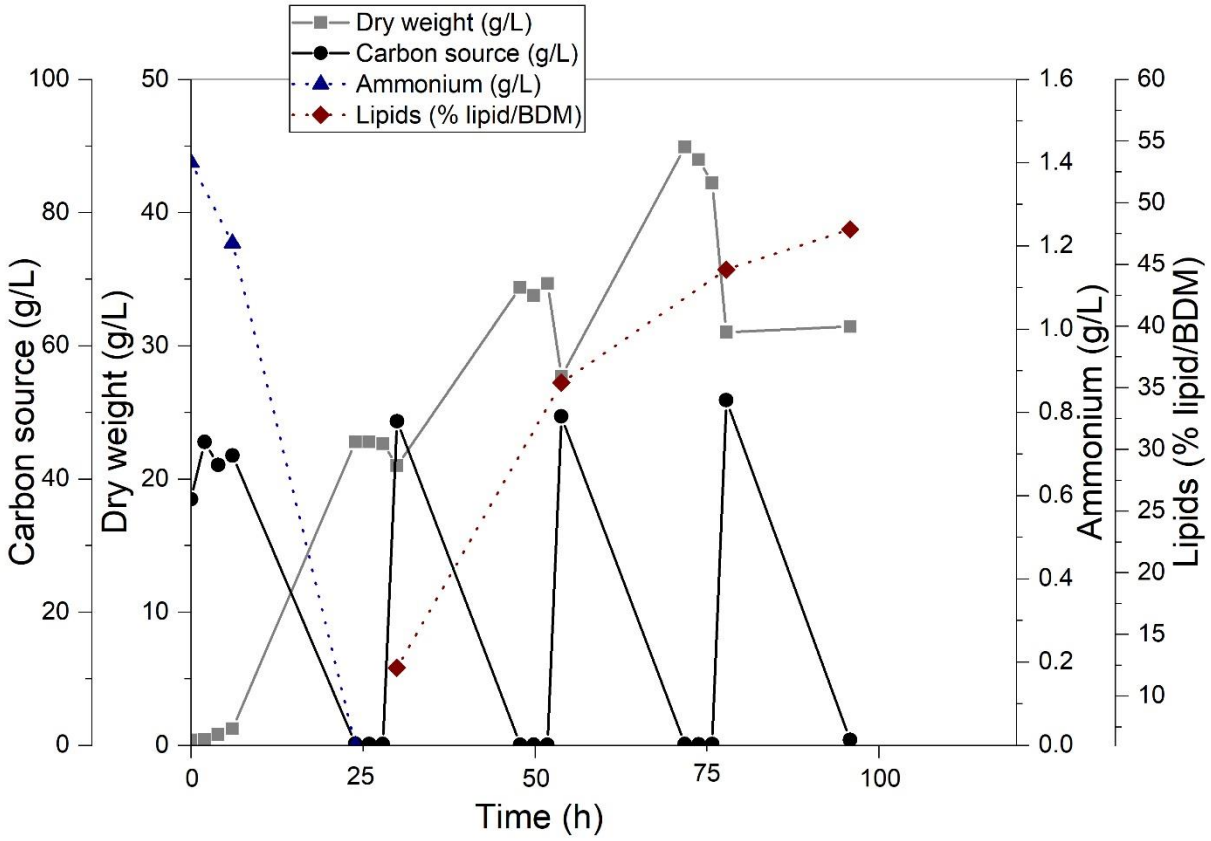

Figure 5.2: Production of single cell oil with the oleaginous yeast *Cryptococcus curvatus* ATCC 20509 in a 2.5 L bioreactor using cellulose fiber hydrolysate from beech wood. All concentrations are given as averages of two independent bioreactor cultivations.

Cellulose fiber hydrolysate proved to be a very suitable carbon source for both biomass and lipid production. Biomass content increased from 0.4 g/L dry biomass to ~45 g/L dry biomass after 72 h of cultivation. After each addition of carbohydrates, a steep increase of biomass was observed. During carbon source consumption, dissolved oxygen concentration dropped quickly (data not shown) until the carbon source was depleted. Since carbon source was limited between feeding cycles, biomass concentration stagnated or was slightly reduced due to sampling and the dilution of culture broth because of feeding. After feeding at 79 h, biomass dropped considerably and did not increase again. Probably due to the high lipid content of the cells at that time, the cell pellet was very soft and cells started to float. Therefore, solid-liquid separation by centrifugation was not efficient and dry biomass concentration might be higher than given in Figure 5.2 at the end of cultivation. Accumulation of single cell oil started after nitrogen depletion at approximately 24 h of cultivation time. Lipid content increased from ~12 % (w/w) to ~48 % (w/w) of dry biomass after 96 h showing the steepest increase between 30 h and 60 h of cultivation time. The final fatty acid profile analyzed via GC revealed a composition of 44.6 % oleic acid (C18:1), 35.1 % palmitic acid (C16:0), 12.6 % stearic acid (C18:0) and 6.2 % linoleic acid (C18:2) (Figure 5.3). Myristic acid (C14:0) has been detected only in trace amounts (0.77 %); all other fatty acids add up to 0.74 %. In total, 256.5 g of carbohydrates have been consumed during the cultivation.

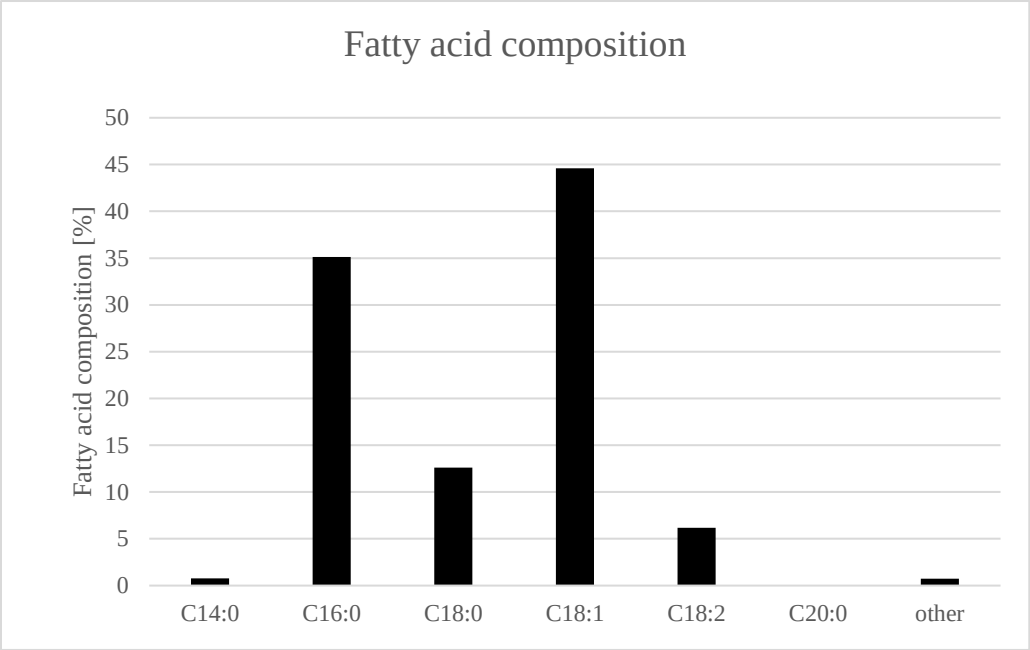

Figure 5.3: Fatty acid composition of the produced fatty acids by C. curvatus with unpurified monosaccharide solution as whole carbon source.

Glucose is likely the most preferred carbon source of *C. curvatus*, however, xylose and cellobiose did not accumulate in culture medium and were completely consumed before the next feeding. The ability of *C. curvatus* to consume xylose and cellobiose as well as a mixture of these two carbon sources with glucose has already been demonstrated by Yu et al. (2014). They demonstrated that cellobiose and xylose can be consumed simultaneously with similar rates, but their consumption is inhibited in the presence of glucose. In a mixture of all three sugars (in pure form) the composition of the mixture had only little impact on the total sugar consumption rate. However, the consumption rate of glucose was considerably higher than the consumption rates of both other sugars. In the presence of a low glucose concentration, i. e. 10 g/L and lower, consumption of xylose and cellobiose seemed to be not affected (Yu et al. 2014). Due to the sampling interval in our study, the exact threshold concentration of glucose cannot be confirmed. However, in preliminary studies in shake flasks it was observed, that xylose accumulated in the medium because glucose was not exhausted before feeding (results not shown). In the study of Yu et al. (2014) the composition of the carbon source did not significantly affect lipid content and composition. Lipid content ranged in all approaches from 38.7 % to 40.3 % lipid per dry biomass with oleic acid being the main fatty acid (41.3-44.8 %) which resembles our results very closely. Compared to cultivations with pure glucose as described by Dillschneider et al. (2014), the results of the present study concerning lipid content (~48 % vs. 45.3 % (w/w)) is very similar. However, concerning the lipid composition (oleic acid: 44.6 % vs. 48.8 %, linoleic acid: 6.2 % vs. 8.6 %, palmitic acid: 35.1 % vs. 18.5 % and stearic acid 12.6 % vs.17.7 %), a higher content of palmitic acid was produced at the expense of oleic acid, linoleic acid, stearic acid and lignoceric acid, which was not detected. The prominent shift in the fatty acid profile towards C16:0 compounds might be explained due to the use of carbohydrate mixtures in our study compared to pure glucose in the study of Dillschneider et al. (2014). However, this is

not supported by the study of Yu et al. (2014) as they also used different carbohydrate mixtures and did only observe a very slight shift in fatty acid profile. Another possible reason might be the presence of by-products in the cellulose fiber fraction due to the organosolv pretreatment, e.g. furfural or HMF. These compounds can be detoxified by the cells, but the mechanisms require the coenzymes NADH or NADPH (Jönsson et al., 2013). For SCO production, high amounts of NADPH are needed, for each addition of a C2 unit 2 NADPH molecules have to be spent (Ratledge, 2004). Therefore, it might be possible, that detoxification of by-products might influence the fatty acid composition of SCO towards shorter fatty acid chain lengths.

Furthermore, yield coefficients for biomass ($Y_{x/s}$) and lipid production ($Y_{p/s}$) were calculated in the presented study to 0.21 g dry biomass per g carbon source and 0.10 g lipid per g carbon source. In comparison, Dillschneider et al. (2014) achieved a higher $Y_{X/S}$ of 0.30 and a slightly higher $Y_{P/S}$ of 0.13 using glucose as sole carbon source. All relevant data of the presented cultivation with cellulose fiber hydrolysate are summarized in Table 5.1 and compared to the study of Dillschneider et al. (2014). It was shown that cellulose fiber hydrolysate from organosolv process can be used as a suitable glucose alternative for the lipid production with *C. curvatus*. Cultivating with pure glucose or a mixture of the three sugars present in our hydrolysate in pure form, results in comparable biomass and lipid content making the hydrolysate a very promising substrate for an emerging bioeconomy. In the presented study, produced fatty acids were succeedingly used after transesterification for enzymatic glycolipid synthesis in DES.

Table 5.1: Summary of measured and calculated process parameters of SCO production with *C. curvatus* ATCC 20509 using cellulose fiber hydrolysate from beech wood in comparison to batch cultivations with pure glucose.

	Cellulose fiber hydrolysate	**Glucose (Dillschneider et al. 2014)***
Cultivation time [h]	96	120
Consumed C-source [g]	256.5	Not given
Lipid content [% (w/w)]	47.9	45.3
Dry biomass conc. [g/L]	44.9	76.2
$Y_{x/s}$ (g biomass per g carbon source)	0.21	0.3
$Y_{p/s}$ (g lipid per g carbon source)	0.10	0.13

5.3.2 Characterizing the DES

To perform a successful biotransformation and for a later optimization of the process, parameters of the DES were identified. In a previous work using the same DES, a pH of 7.5 and water content of 5.4 % were already determined (Siebenhaller et al., 2017a). In addition to these data, a water activity of 0.101 was measured. To a certain degree, the presence of free water molecules helps to maintain the enzymes` essential hydrate shell. Therefore, a small amount of water should not abet the hydrolysis of formed

glycolipids, the water content is more likely to support the reaction which was already reported in various studies (Erwann Durand et al., 2013; Guajardo et al., 2017a).

The viscosity of sugar-based DES is very high and depends on the sugar used, the molar ratio, the water content and the temperature. A similar DES to the one used here, CC:Glu in a molar ratio of 1:1, had a viscosity of 34,400 mPa*s at 50 °C (Maugeri et al., 2012). The DES is this study consisting of one part CC and one part sugar (glucose and xylose) is less viscous with 2.540 mPa*s. A reason could be the water content of 5.4 %, which will reduce the viscosity of a DES (Shah and Mjalli, 2014). Further, a nearly ten times higher viscosity of 23.340 mPa*s was determined at 25 °C, which also indicates the highly temperature depending viscosity of DES (Maugeri and Domı, 2012; Stefanovic et al., 2017).

5.3.3 Detection of synthesized products via thin-layer chromatography

For the production of glycolipids, a DES consisting of choline chloride and purified cellulose hydrolysate fraction was prepared and produced FAMEs from SCO and iCalB were added for the synthesis of sugar esters. As a fast analysis of the crude glycolipid extract, TLC was performed to detect expected sugar esters (Figure 5.4).

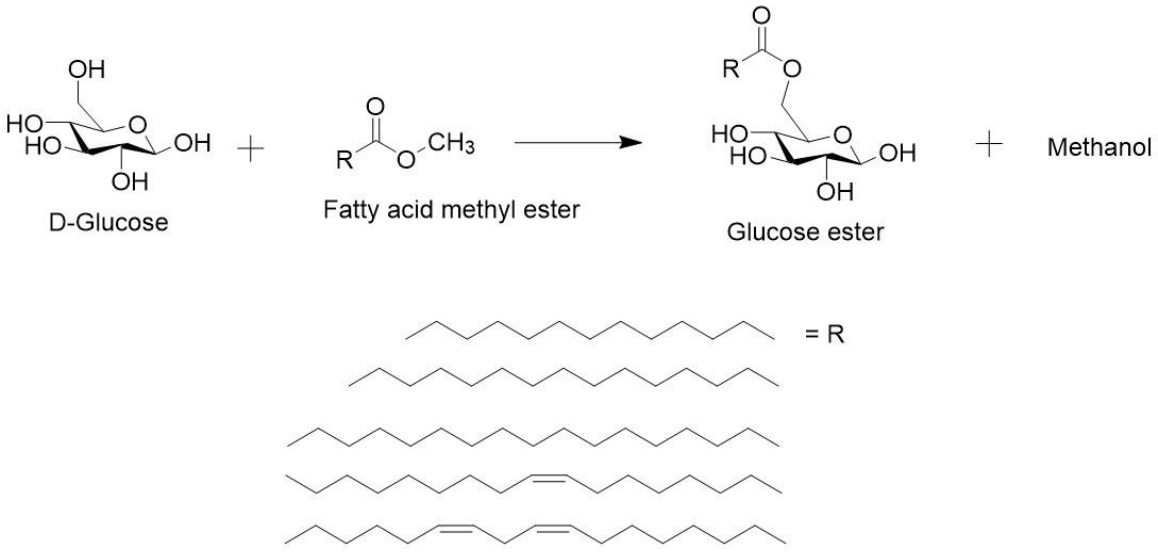

Figure 5.4: Lipase catalyzed transesterification reaction in the DES with sugar (e.g. glucose) and a methylated fatty acid ester produced from SCO (methyl myristate, methyl palmitate, methyl stearate, methyl oleate and methyl linoleate). The reaction leads to a sugar ester and methanol as side product. Methanol can enter the gas phase and push the reaction forwards or inhibit the enzyme.

Synthesis products in this extract showed various spots (Figure 5.5). The spot with the retention factor (RF) 0 is a mixture of glucose (dark gray) and xylose (yellow) of the DES, verified by a standard of the pure sugars (data not shown). The light grey-yellowish spots between RF 0.05 and 0.42 indicate the formation of different glucose- and xylose-esters. The height of each spot depends on the chain length and saturation state of the sugar esters carbonyl chain. In this area, a clear spot with a RF 0.08 seems to

be the main product of the enzymatic reaction. Above, two dark purple spots with RF 0.75 and 0.92 are visible. These spots are fatty acid methyl esters which were not consumed during the synthesis reaction.

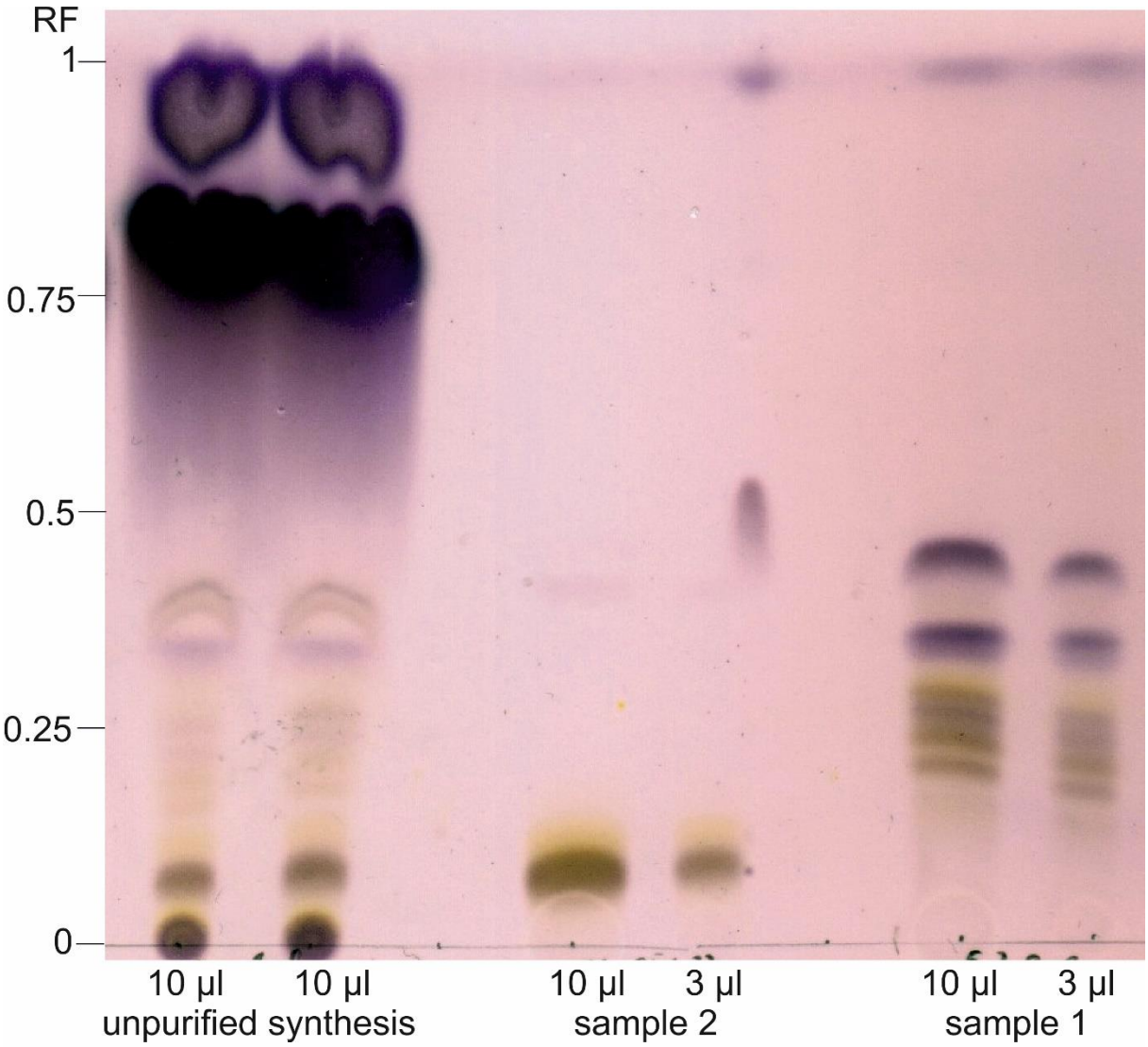

Figure 5.5: Thin layer chromatography of synthesis products from the enzymatic reaction of microbial fatty acid methyl esters and beech wood cellulose fiber hydrolysate sugars catalyzed by iCalB in a DES reaction system. The crude synthesis extract, the purified and unified sample 1 and 2 were applied. After the run, the plate was dyed with anise aldehyde solution. Colored spots indicate the formation of various sugar esters.

To get further information and for further analysis, the crude extract was purified and fractionated via flash chromatography. Sample 1 as well as sample 2 show the same spot pattern according to TLC analysis. Therefore, sample 1 and 2 were unified and concentrated. Sample 3 resulted in the clearest spot and was therefore regarded as being the purest fraction of the main product and, consequently, was used for NMR analysis.

In both purified fractions, only trace amounts of FAMEs are visible at the running front. Sample 2 contains the main product, a clear grey colored spot portending to a glucose-ester with a slight yellow aura, indicating for a xylose-ester. The comparison of the height of these spots to known sugar esters led to the assumption that they are sugar-monoesters (Siebenhaller et al., 2016). The analysis of sample 1

showed a grey-yellow striped pattern between RF 0.17 and 0.25. The there-in contained products have either a much longer hydrophobic carbonyl chain, which is unlikely with the FAME-composition applied in our study, or two or more fatty acids are bound to the polar sugar.

During transesterification of a FAME and a sugar, methanol is formed as a side-product. Methanol can enter the gas phase and push the reaction towards acylation but in a sealed reaction vessel and with a boiling point of 64 °C, most of the methanol will likely stay in the DES. However, methanol has been reported to inhibit the activity of the lipase (Coulon et al., 1996).

To overcome that, the reaction efficiency might be improved by increasing the temperature to > 65 °C, taking care that the enzyme remains still active. By using other fatty acids like vinyl esters, acetaldehyde will be formed as side product, which evaporates much easier at lower temperatures (Bornscheuer and Yamane, 1995). However, the vinylation reaction is much more complicated than the methylation of fatty acids. Another method of optimization would be to carry out the process at reduced pressure to remove side products immediately and shift the reaction equilibrium (Ducret et al., 1995).

The successful formation of mono- or polyacylated sugars in this DES system with the used iCalB and commercial available vinyl esters was already shown (Siebenhaller et al., 2017a); the synthesis with sustainable fatty acid methyl esters for the production of glycolipids was verified by subsequent mass spectrometry and NMR analysis.

5.3.4 Mass analysis of the formed sugar esters

The successful enzymatic synthesis of sugar esters were affirmed by three different mass analysis procedures. First, with the TLC-MALDI-ToF technique it was possible to assign product spots of sample 1 and 2 to a detected *m*/*z* value. In the lane of sample 1, masses were detected in three areas. Surprisingly, in area 1 (A1: RF 0.59-0.67) and area 2 (A2: RF 0.72-.082) the same *m*/*z* values were determined (Appendix 13 and Appendix 14). Several *m*/*z* values of sodium adducts of glucose-di-esters with the following fatty acid pairs were identified: *m*/*z* 680.04 (myristic acid/stearic acid or more likely palmitic acid/palmitic acid), *m*/*z* 706.47 (palmitic acid/oleic acid), *m*/*z* 708.09 (palmitic acid/stearic acid), *m*/*z* 727.66 (linoleic acid/linoleic acid) and *m*/*z* 734.10 (stearic acid/oleic acid). Throughout the entire area 3 (A3: RF 0.86-0.88), only *m*/*z* 686.0 was detected, which corresponds to a protonated glucose, acylated with palmitic acid and stearic acid. Due to the high number of potentially formed sugar-di-esters, more possible combinations are feasible. Therefore, it is possible, that *m*/*z* 680.04 corresponds to a protonated xylose, which is linked to 2 oleic acids, or to stearic acid/linoleic acid.

By applying sample 2 onto the silica plate and subsequent analysis, two mass areas (a1; RF 0.33-0.45 and a2: RF 0.84-0.89) were found. In a1, sodium adducts of the more polar reaction products glucose palmitate (*m*/*z* 441.66) glucose oleate (*m*/*z* 467.71) and glucose stearate (*m*/*z* 469.74) were detected. a2

indicates the presence of more hydrophobic glucose-di-esters, which was expected due to the TLC separation. One of these di-ester corresponds to a protonated glucose with palmitic acid and stearic acid (m/z 686.04), the other di-ester matched to the sodium adduct of a glucose, linked to 2 linoleic acids (*m*/*z* 727.61). These results show that the enzymatic synthesis was successful.

Due to possible matrix and silica problems by the detection of molecules with low masses, ESI-Q-ToF MS were performed to verify the results.

By applying ESI-Q-ToF MS to sample 2 various sugar-mono-esters were identified. The synthesis of glucose palmitate (M_{GP} = 418.29 Da) was verified through the occurrence of ions at *m*/*z* 441.27 (M_{GP} + Na^+), *m*/*z* 436.30 (M_{GP} + NH_4^+), *m*/*z* 401.26 ($M_{GP} - H_2O + H^+$) and *m*/*z* 383.23 ($M_{GP} - 2H_2O + H^+$). The same adducts of glucose-oleate (M_{GO} = 444.31 Da) were detected at *m*/*z* 467.27, 462.32, 427.26 and 409.27. Further, glucose stearate (M_{GS} = 446.32 Da) and glucose linoleate (M_{GL} = 442.29 Da) was detected through the ions $M_{GS} - H_2O + H^+$ (*m*/*z* 429.29) respective $M_{GL} - H_2O + H^+$ (*m*/*z* 425.25). Thus, it was possible to detect sugar esters of the most common FAMEs. In addition, a MALDI-ToF MS of sample 1 was accomplished to verify the occurrence of sugar-di- or poly-ester with higher masses and to overcome possible problems of the TLC-silica. There, masses corresponding to synthesized sugar-di-esters were detected, too. Sodium adducts with *m*/*z* 733.56, *m*/*z* 731.55, *m*/*z* 705.52 and *m*/*z* 679.55 indicates the formation of glucose-di-ester with the following acylated fatty acid pairs: stearic acid/oleic acid (712.58 Da), stearic acid/linoleic acid (708.55 Da), oleic acid/oleic acid (708.55 Da), palmitic acid/oleic acid (682.53 Da) and palmitic acid/palmitic acid (656.52 Da). At *m*/*z* 699.5, the sodium adducts of a di-acylated xylose with oleic acid/linoleic acid (676.53 Da) shows, that xylose was used as a substrate, too.

The results of the mass analysis confirmed the formation of glycolipids in the used DES with FAMEs.

Beside the mentioned products, other sugar-mono- and di-esters might be formed. Since it is shown in literature, it cannot be excluded that sugar-poly-ester were also synthesized in this reaction system, even if none were detected (Siebenhaller et al. 2016). Since many products or their adducts have the same mass, it is nearly impossible to distinguish between them. It can only be stated that, based on the FAME-mixture used and the amount of fatty acids contained therein, some sugar esters will be probably less common than others. Sugar esters with the four main fatty acids (palmitic acid, stearic acid, oleic acid and linoleic acid) were detected; therefore, the used FAME-Mix is a suitable substrate for this reaction.

It was possible to identify some xylose esters in fraction 1 via MALDI-ToF MS, but these are much rarer than glucose ester. Since glucose occurs 4-times more often than xylose in the purified and dried cellulose fraction, this is justified.

Mass spectra are shown in Appendix 15 to Appendix 19.

5.3.5 Identification of sugar ester structure by NMR

The purified and fractionated sample 3 of an iCalB catalyzed synthesis reaction in a DES, based on lignocellulosic sugars, with FAMEs, was investigated with one and two-dimensional ^{1}H and ^{13}C NMR spectra. Glucose was identified through one clear major carbohydrate system, starting from the anomeric protons of the ^{1}H COSY and ^{13}C HMBC spectra. Cross peaks between the two protons attached at the glucoses` C6 at 4.23 and 4.35 ppm with the lipid carbonyl at 174.03 ppm in the $^{1}H^{13}C$ HMBC indicates, that glucose was acylated with fatty acid methyl esters (Table 5.2). Due to some impurities and the used mixture of fatty acid methyl esters, it was not possible to clearly identify which FAME is connected to the glucose molecule.

Table 5.2: Chemical shifts of the main product, present in the purified and fractionated sample 3. The shifts indicate a sugar system, identified as glucose with acylation at the C6-atoms.

glucose	C shift (ppm)	H shift (ppm)
-C^{1}H-O-	92.6	5.21
-C^{2}H-	72.6	3.47
-C^{3}H-	74.0	3.76
-C^{4}H-	70.2	3.36
-C^{5}H-	69.6	3.97
-C^{6}H- (acylated C'174.03)	63.7	4.23
-C^{6}'H- (acylated C'174.03)	63.7	4.35

A view on the structure of glucose shows, that less steric effects affect the glucoses exposed C6-atom, so that this acylation site seems to be favored. This was also observed in previous work by our group (Siebenhaller et al., 2017a).

This result and the mass analysis data confirm that sugar esters have been produced during the synthesis process.

However, in the analyzed sample no traces of xylose or acylated xylose were detected. This can be explained by the fact that xylose occurs over 4 times less frequently than glucose and that xylose esters may have been removed during purification of the analyzed fraction. However, in the mass analysis of unified fraction 2, xylose or its esters were not detected either.

5.4 Conclusions

The presented study demonstrates the possibility to use sustainable lignocellulosic biomass as sole substrate for the production of high valuable fatty acid sugar esters. It was shown, that cellulose fiber hydrolysate from organosolv process is a suitable substrate for SCO production with *C. curvatus* being completely comparable to pure sugars regarding biomass and lipid content. Subsequently, a DES reaction system based on sugars of the hydrolysate was established, acting simultaneously as reaction media and together with the produced fatty acids as substrate for the enzymatic sugar ester synthesis. The successful formation of sugar esters were proven by NMR analysis. Further, several sugar (di-)esters like glucose palmitate or oleate and glucose di- linoleic acid were detected by various MS experiments. For a better understanding of the reaction media characteristics of the used DES were determined.

5.5 References chapter 5

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6 Usage of natural sugar containing liquids as substrate for the enzymatic sugar ester synthesis

This chapter is mainly based on the publication

Lipase-Catalyzed Synthesis of Sugar Esters in Honey and Agave Syrup

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Frontiers in Chemistry

Volume 6, Article 24

Published: 12 February 2018

DOI: 10.3389/fchem.2018.00024

Authors' contribution to this publication

Sascha Siebenhaller designed the study and the manuscript, conducted experiments and analyzed the results. He supervised Julian Gentes.

Julian Gentes carried out experiments like synthesis reactions, TLC detection, HPLC measurements, purification and characterization of formed glycolipids.

Alba Infantes measured the pH values and proofread the manuscript.

Claudia Muhle-Goll performed the NMR measurements and help to analyze them. Write and proofread parts of the NMR sections.

Frank Kirschhöfer and **Gerald Brenner-Weiss** helped to perform the ESI-ToF experiments. Write and proofread parts of the ESI-ToF sections.

Katrin Ochsenreither and **Christoph Syldatk** supervised the project and provided scientific input for this study.

6.1 Abstract

In nature liquids with similar compositions and properties like deep eutectic solvents occur. Some of these substances consist of only a few ingredients, others are complex mixtures. Two of them, honey and agave nectar, are not considered as natural deep eutectic solvents, but they have roughly similar properties to some known DES. Honey is a mixture of over 180 ingredients, of which over 80 % are carbohydrates, mainly glucose and fructose. In contrast, agave nectar consists of much fewer ingredients, but the major components are fructose and glucose, too. Next to their usage as sweetener or as food additives, especially honey is used since ancient times in dermatology and skin care and is still an important and often used ingredient for cosmetics and some medical ointments today. By working with established high sugar content DES with similar properties and compositions, honey and agave nectars look like being a natural suitable substrate for the enzymatic synthesis of glycolipids. Therefore, in this chapter important characteristics of the herein used honey and agave syrup were determined and compared with other available types. Subsequently, an enzymatic transesterification of four fatty acid vinyl esters was accomplished in ordinary honey and agave syrup, which act simultaneously as substrate and media. Notwithstanding of the high water content for transesterification reactions of honey and agave nectar, the successful sugar ester formation was proven by thin-layer chromatography and compared to a sugar ester which was synthesized in a conventional deep eutectic solvent. For a clear verification of the sugar esters, mass determinations by ESI-Q-ToF experiments and a NMR analysis were done. These environmentally friendly produced sugar esters have the potential to be used in cosmetics or pharmaceuticals, or to enhance their effectiveness.

6.2 Materials and Methods

6.2.1 Materials

Lipase B from *Candida antarctica*, immobilized on acrylic resin (iCalB) and choline chloride (98 %) were purchased from Sigma-Aldrich (Germany). Commercially available honey ("Flotte Biene, Obstblütenhonig", Langnese Honig GmbH & Co. KG, Germany) and agave syrup ("Agaven Dicksaft Fruchtsüße", dmBio, dm-drogerie markt GmbH + Co. KG, Germany) were used as substrates. All used fatty acid vinyl esters were acquired from Tokyo Chemical Industry Co. Ltd (TCI-Europe, Belgium). If not stated otherwise, all other chemicals were purchased from Carl-Roth (Germany).

6.2.2 Methods

6.2.2.1 Enzymatic synthesis of glycolipids

The enzymatic synthesis of glycolipids was accomplished as follows: 20 mg of iCalB, 200 µL of a fatty acid vinyl ester (vinyl palmitate, vinyl laurate, vinly decanoate or vinyl octanoate) and 2.5 mL of honey or agave syrup were filled in a 5 mL Eppendorf Tube. After rigorous shaking, the reaction was carried out in a rotator with vortex mixer in program U2 at 50 rpm (neoLab, Germany) at 50 °C for 48 h. As a control, reactions without enzyme or without a fatty acid vinyl ester were made.

As a reference, fructose- and glucose based glycolipids were synthesized in a conventional DES. For this, choline chloride and the corresponding sugar were mixed in a molar ratio of 1:1 at 100 °C under constant stirring until a liquid was formed. Afterwards, lipase and fatty acid vinyl ester were added and the reactions were performed as mentioned above.

6.2.2.2 Extraction of glycolipids and purification by flash chromatography

Prior to analysis, synthesized glycolipids were extracted from reaction media by adding 2 mL of warm water and rigorously shaking the obtained mixture. After further addition of 3.5 mL ethyl acetate and shaking for 45 s, a glycolipid containing organic phase was formed. This was collected and used for thin-layer chromatography and purification.

For purification of synthesis products by flash chromatography (Reveleris Prep, Büchi Labortechnik GmbH, Germany), six identical extracts were unified and concentrated to a volume of approximately 4 mL. The concentrated phase was applied to a Revelersis HP Silica 12 g column at a flow rate of 30 mL/min, a chloroform:methanol gradient was used for the separation of synthesis products as follows: 0 % methanol to 10 % in 1.8 min, holding the gradient for 7.1 min. Afterwards, increase to 15 % in 3.6 min and to 100 % in 1.8 min. It was hold for 1.8 min to remove all sugars. Product peaks were observed

by an evaporative light scattering detector (Treshold: 20 mV, Sensitivity: low) and fractionated. Fractions were analyzed via thin-layer chromatography (TLC) and subsequently used for MS and NMR analysis.

6.2.2.3 Detection of glycolipids via thin-layer chromatography

For qualitative analysis of formed glycolipids, 10 µL of the crude extracts were spotted onto a silica gel TLC plate as stationary phase (Alugram SIL G, 60 Å, Macherey-Nagel GmbH & Co. KG, Germany). The mobile phase consists of chloroform:methanol:acetic acid (65:15:2, by vol.) to separate the synthesized compounds (Pöhnlein et al., 2015b). Visualization of the different glycolipids was accomplished by incubation of the TLC plate in a dyeing solution. The dyeing solution consists of anise aldehyde: sulfuric acid:acetic acid (0.5:1:100, by vol.). After incubation, the TLC plate was heated by a 200 °C hot air stream for approximately 5 min.

6.2.2.4 Determination of the accurate masses via electrospray ionization quadrupole time of flight mass spectrometry

The accurate masses of the synthesis products, which were separated and purified by flash chromatography, were determined with an ESI-Q-ToF MS system (Q-Star Pulsar i, AB SCIEX, Germany) equipped with an electrospray ionization (ESI) source. All measurements were carried out in the positive mode within a mass range from *m*/*z* 50 to *m*/*z* 800 using the activated "enhance all" setting. Before measurement, the samples were diluted 1:5 in a mixture of 10 mM ammonium acetate and methanol (1:1, by vol.) and continuously infused via a syringe pump at a flow rate of 10 µL/min.

The ion source voltage was set to 4,800 V, declustering potential to 30 V and focusing potential to 100 V. As nebulizer and curtain gas, nitrogen gas 5.0 was used in all experiments.

Data acquisition and processing were performed using the Analyst QS 1.1 software (AB SCIEX, Germany).

6.2.2.5 Structural elucidation of sugar esters via nuclear magnetic resonance spectroscopy

For NMR spectroscopy, 12 mg of purified sugar octanoates based on honey and 13 mg based on agave syrup were dissolved in 0.6 mL $CDCl_3$ and d6-acetone (4:1, by vol.). 1D ^{1}H NMR spectroscopy and 2D ^{1}H–^{1}H correlation spectroscopy (COSY), ^{1}H–^{13}C heteronuclear single quantum coherence spectroscopy [clip-HSQC (Enthart et al., 2008)], and heteronuclear multiple-bond correlation spectroscopy (HMBC) were recorded with a Bruker AVANCE II +600-MHz spectrometer (Bruker AG, Germany) equipped with a BBI probe head. Recorded spectra were analyzed with Topspin 3.2 (Bruker AG). Intensities were

measured from a 1D ^{1}H spectrum acquired with a sixteen scan and four dummy scans. Chemical shifts are referenced to the ^{1}H and ^{13}C resonance of tetramethylsilan.

6.2.2.6 Characterization of the used honey and agave syrup

The water content of honey and agave syrup was measured via Karl Fischer titration (TitroLine 7500 KF trace, SI Analytics, Germany). Before measuring, the titrator was tested with a water standard (Merck Millipore, Germany).

The water activity aw of honey and agave syrup was determined with an AquaLab CX-2 at 22 °C (Decagon Devices, USA).

After calibrating, the pH was directly measured in honey and agave syrup and as a 10 % dilution (w/v) (SenTix® Mic, Xylem Analytics, Germany).

The two main carbohydrates, fructose and glucose, were quantified by HPLC (Agilent 1100 Series, Agilent Technology, Germany) with a Rezex ROA organic acid H+ (8 %) column (300 mm length, 7.8 mm diameter) and a Rezex ROA organic acid H+ (8 %) guard column (50 mm length, 7.8 mm diameter) from Phenomenex (Phenomenex Ltd, Germany) as described in Dörsam et al. (2017). Separation was performed with 5 mM H_2SO_4 for 45 min and a flow rate of 0.5 mL/min under isocratic conditions at 50 °C column temperature. Carbohydrates were detected via a refractive index detector (Agilent 1200 series, Agilent Technology, Germany).

Quantification of the sugars were performed by using three different dilutions of honey or agave syrup (0.3 mg/mL to 1 mg/mL) and an external 10-point calibration curve for each component from 10 mg/l to 500 mg/l.

All measurements were made in triplicates.

6.3 Results and discussion

6.3.1 Characterization of the natural products

For characterizing the used sugar-containing natural products - honey and agave syrup - the contents of the two major carbohydrates fructose and glucose were determined by HPLC.

The used honey sample consisted of 0.36 g glucose and 0.46 g fructose per g honey and reflects therefore the average concentration of these sugars (40.9 % fructose and 35.7 % glucose; Table 6.1). Since honey is a natural product, the composition and total amount of carbohydrates vary between 80 and 83 %, depending on the pollen source and climate (Ajibola et al., 2012; United States Department of Agriculture, 2016).

The used agave syrup has an 8 % higher carbohydrate concentration compared to honey, divided in 0.7 g fructose and 0.19 g glucose per g agave syrup. In a study from 2012, Willems *et al.* compare the major carbohydrates of 20 pure agave syrups. There, the mean value of fructose is 84.3 % and 8.3 % for glucose, which is similar to the measured data. Additional carbohydrates in honey and agave syrup, like the disaccharides maltose or sucrose were not determined via HPLC since they usually occur only in small quantities.

With a density of approximately 1.4 g/cm^3, the volume of 2.5 mL of honey or agave syrup used for the synthesis reactions corresponds to a mass of 3.5 g of which over 2.8 g is sugar; this is equal to 15.5 mmol. Therefore, the applied amount of fatty acid vinyl esters (200 µL) is the limiting substrate in all reactions, because this amount corresponds, depending of the fatty acid vinyl ester, to a range between 0.6 and 1.03 mmol. In an earlier study by our group it was shown that the yield in a sugar-based DES under similar conditions is around 5 %, which corresponds to a consumption of less than 1 % of the used sugar (Siebenhaller et al., 2017a). This suggests that the amount of honey or agave syrup used in this non-optimized process is in excess and therefore, does not limit sugar ester formation.

The determined water content of both substrates was roughly the same, with 17 % in honey and 15 % in agave syrup and should therefore only have a small impact of the synthesis reaction. The average water content for honey given in literature is 17.2 % (Ajibola et al., 2012), for agave syrup it is 30 % (José Luis Montañez Soto, 2011). However, it must be noted that agave syrup is often processed before it is used as natural sweetener, which could explain the difference to the measured value.

Beckh *et al.* determined the water activity of 70 different types of honey (Beckh et al., 2004). 31 were fluid with an a_w ranging from 0.52 to 0.64. The used honey for the synthesis of glycolipids has a water activity of 0.56, and is therefore in the a_w range for honey. The used agave syrup has an a_w of 0.64 and is near the value of 0.69 which Soto measured for agave syrup (José Luis Montañez Soto, 2011).

The pH of the measured samples was 3.4 for honey and 3.9 for agave syrup. The pH for honey coincides with other literature values. There it is often stated as pH 3.6, with variations between pH 3.3 and 7 (Kwakman et al., 2010; Wahdan, 1997). The pH in pure agave syrup was pH 3.9, and in the diluted solution it was pH 4.3, which is in the range of pH 4.1 to 5.5 for diluted agave syrup (Willems and Low, 2012).

The characterization of the natural products was necessary in order to give a general statement on the reproducibility of the reaction. Since all the parameters determined are correlating well with known literature values, it can be implied that our results are transferable to other honey and agave syrup samples.

Table 6.1: Summary of the characterization of herby used honey and agave syrup.

	Honey	Agave syrup
Glucose content [g/g]	0.36 ± 0.02	0.19 ± 0.01
Fructose content [g/g]	0.46 ± 0.04	0.70 ± 0.02
Water content [%]	17 ± 1	15 ± 1
Water activity	0.56	0.64
pH pure	3.4	3.9
pH diluted 1:10 (w/v)	3.61	4.29

6.3.2 Analysis of the synthesized products

The extracts of all reactions in honey and agave syrup with the four tested fatty acid vinyl esters contained several compounds as visualized by positive spots on the dyed TLC plate (Figure 6.1). To verify that these spots were formed by the enzymatic synthesis, negative controls without enzyme were compared with the synthesis reactions (Appendix 20 and Appendix 21). Thereby, it was clearly shown that without the addition of enzyme formation of glycolipid is not possible.

The main spots of all extracts have a RF between 0.56 and 0.61. These small difference in the RF values of the spots are depending on the length of the carbonyl chains which is linked to the sugar by influencing the polarity of the whole molecule. For example using vinyl palmitate, the substrate with the longest carbonyl chain, the main spot has a RF of 0.61, and with vinyl octanoate it slightly decreases to 0.56 (Table 6.2).

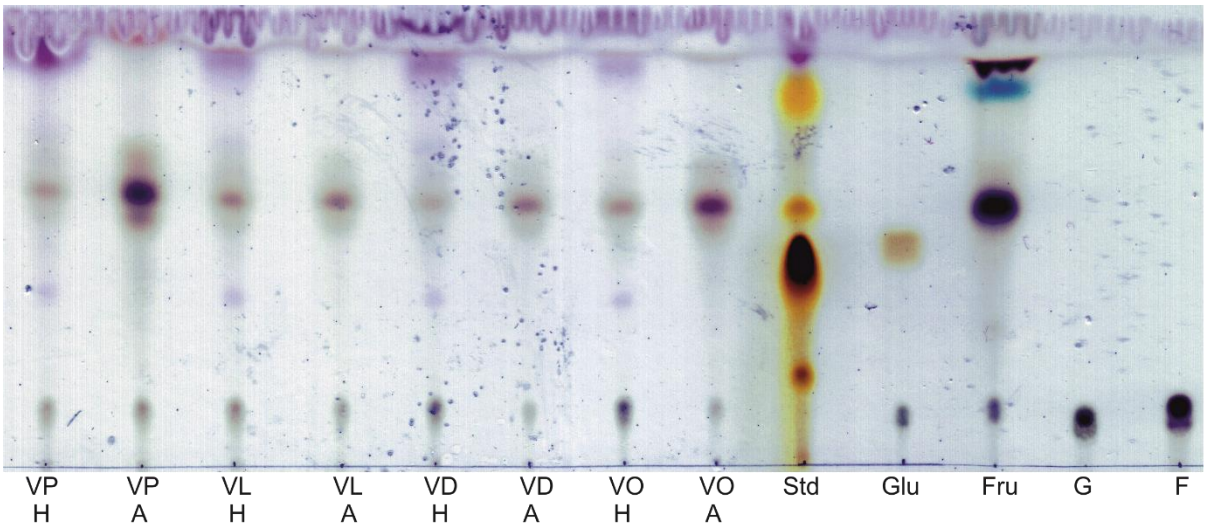

Figure 6.1: Visualization of synthesized glycolipids in honey and agave syrup after dying with an anise aldeyhde solution. 10 µL of the extracts and 4 µL of the standards were spotted on the TLC plate. VP = vinyl palmitate; VL = vinyl laurate; VD = vinyl decanoate; VO = vinyl octanoate; H = synthesis in honey; A = synthesis in agave syrup; Std = lab intern rhamnolipid standard; Glu = glucose based DES with VO; Fru = fructose based DES with VO; G = glucose, solved in an ethanol-water mixture; F = fructose in an ethanol-water mixture.

Further spots are visible in the extract with honey as substrate. With RF values between 0.87 and 0.93, some of these spots can be located directly in the front of the mobile phase (VL H, VD H andVO H). These indicates the formation of di- or poly-acylated sugars, which also have been observed several times in prior experiments (Siebenhaller et al., 2016). There are also very weak spots at RF values between 0.37 and 0.39, which can not be explained.

With agave syrup as substrate, the vinyl palmitate extract showed some unique spots. There is one with a high RF of 0.96 which pointed to a di- or poly-acylated sugar. Fruther, a triple spot between RF 0.55 and 0.68 is visible. We hypothesize that the triple spot is a sugar esters which might have several configurations, leading to a slightly different polarity, resulting in a different running behavior. In the other reactions this triple spot is not clearly visible.

The visualization of extracted reaction products of glucose-based DES shows only one main spot, whereas the products of fructose-based DES shows two additional spots with higher RF values, of which the light blue one only occurs in this sample. Therefore, this blue spot is no synthesis product but might be a pH-depending configuration of the fructose molecule in a DES, since it does not occur in the pure fructose control. When comparing the synthesis products in honey and agave syrup with the fructose-based DES and vinyl octanoate as substrate, the deduced product spots have the same RF values (0.56 and 0.12, respectively). However, the synthesis product of the glucose-based DES has a lower RF of

0.47, which can not be seen in any other synthesis reaction. This may indicate that fructose might be more favored by the used lipase rather than glucose.

Furthermore, in all reaction extracts a spot at RF 0.12 is visible, corresponding to free fructose or glucose, due to the similar running characteristics of fructose and glucose.The lab internal rhamnolipid standard (Std) was used as a control for TLC separation and dyeing of the thin-layer plates.

Table 6.2: RF values of all visible spots and their corresponding possible compounds.

Sugar source	Fatty acid ester / carbonyl chain	Main RF values	Possible compounds
Honey	Vinyl palmitate / C14	0.93	Sugar-di- or poly-palmitate
		0.61	Fructose-mono-palmitate
		0.39	Unknown, occurs in negative controls, too
		0.12	Fructose
Agave syrup	Vinyl palmitate / C14	0.96	Sugar-di- or poly-palmitate
		0.68	Fructose-mono-palmitate
		0.60	Fructose-mono-palmitate
		0.55	Fructose- or glucose-mono-palmitate
		0.12	Fructose
Honey	Vinyl laurate / C12	0.90	Sugar-di- or poly-laurate
		0.58	Fructose-mono-laurate
		0.39	Unknown, occurs in negative controls, too
		0.12	Fructose
Agave syrup	Vinyl laurate / C12	0.58	Fructose-mono-laurate
		0.12	Fructose
Honey	Vinyl decanoate / C10	0.89	Sugar-di- or poly-decanoate
		0.57	Fructose-mono-decanoate
		0.38	Unknown, occurs in negative controls, too
		0.12	Fructose
Agave syrup	Vinyl decanoate / C10	0.57	Fructose-mono-decanoate
		0.12	Fructose
Honey	Vinyl octanoate / C8	0.87	Sugar-di- or poly-octanoate
		0.56	Fructose-mono-octanoate
		0.37	Unknown, occurs in negative controls, too
		0.12	Fructose
Agave syrup	Vinyl octanoate / C8	0.56	Fructose-mono-octanoate
		0.12	Fructose
DES Glucose	Vinyl octanoate / C8	0.47	Glucose-mono-octanoate
		0.10	Glucose
DES Fructose	Vinyl octanoate / C8	0.88	Sugar-di- or poly-octanoate
		0.83	Unknown, occurs in negative controls, too
		0.56	Fructose-mono-octanoate
		0.12	Fructose
Glucose Std		0.10	Glucose
Fructose Std		0.12	Fructose

6.3.3 Determination of glycolipid masses via electrospray ionization quadrupole time-of-flight mass spectrometry

Purified sugar ester fractions were used for the validation of the successful enzymatic synthesis of glycolipids in honey and agave syrup, using vinyl octanoate as a representative substrate. The used fractions had a RF value of 0.55; the glucose- or fructose octanoate had a calculated molar mass of 306.168 Da ($M_{G/F}$). The presence of ions at *m*/*z* 289.312 ($M_{G/F}$ - H_2O + H^+), the occurrence of the ammonium adduct at *m*/*z* 324.360 ($M_{G/F}$ + NH_4^+) and the sodium adduct at *m*/*z* 329.311 ($M_{G/F}$ + Na^+) verifies the formation of glucose- or fructose octanoate ($M_{G/F}$), both in honey and agave syrup (Table 6.3 as well as Appendix 23 and Appendix 24). Due to the identical mass of glucose octanoate and fructose octanoate, it can not be conclusively clarified which sugar is connected to the octanoate via mass spectrometry.

The *m*/*z* 229.205, which occurs in the honey fraction, can be explained by the loss of two CH_2O molecules and a water cleavage from the formed sugar octanoate (Lie et al., 2015).

The smaller observed masses (*m*/*z* 109.139, 127.135 and 145.158) did not correlate to a formed sugar ester. Therefore, further MS experiments were carried out with pure glucose in which these masses also occurred, indicating for sugar cleavage products.

Hitherto, the three detected masses between *m*/*z* 189.196 and *m*/*z* 224.234 can not be assigned to an expected product. These masses could match to one of the other ingredients of agave syrup.

In unpurified samples of a synthesis reaction in honey and in agave syrup with vinyl octanoate, higher masses with *m*/*z* 415.087 and *m*/*z* 450.107 are occurring in addition to the above mentioned *m*/*z* values. These values are matching to synthesized sugar di-octanoates ($M2_{G/F}$) with a calculated molar mass of 432.272 Da. The observed *m*/*z* values are $M_{2G/F}$ - H_2O + H^+ and the ammonium adduct $M_{2G/F}$ + NH_4^+. This is consistent with previous results of synthesis reactions in deep eutectic solvents, in which the formation of mono- and di-acylated sugars were observed via ESI-Q-ToF-MS, too (Siebenhaller et al., 2017a). Due to their separation during flash chromatography, sugar di-octanoates were only detected in the unpurified synthesis extracts.

By using other fatty acids, like the shorter vinyl hexanoate (278.14 Da) or longer vinyl laurate (362.23 Da), corresponding masses of formed sugar ester adducts were also detected (data not shown).

Table 6.3: Observed m/z values during ESI-Q-ToF experiments after flash purification of fraction 8 of with vinyl octanoate in honey, respective fraction 9 in agave syrup. Masses compare to glucose- or fructose octanoate with a calculated molar mass of 306.168 Da (MG/F) and to glucose- or fructose-di-octanoate with 432.272 Da (M2G/F).

Observed *m/z* value	Calculated *m/z* value	Sample / Fraction	Corresponding fragment
109.139	109.023	Honey + V-Oct / 8	Sugar cleavage products
127.135	127.033	Honey + V-Oct / 8	
145.158	145.043	Honey + V-Oct / 8	
229.205		Honey + V-Oct / 8	$M_{G/F}$- H_2O - 2 CH_2O + H^+
271.290	271.145	Honey + V-Oct / 8	$M_{G/F}$ - 2 H_2O + H^+
289.318	289.158	Honey + V-Oct / 8	$M_{G/F}$ - H_2O + H^+
324.374	324.202	Honey + V-Oct / 8	$M_{G/F}$ + NH_4^+
329.336	329.158	Honey + V-Oct / 8	$M_{G/F}$ + Na^+
109.139	109.023	Agave + V-Oct / 9	Sugar cleavage products
127.135	127.033	Agave + V-Oct / 9	
145.158	145.043	Agave + V-Oct / 9	
189.196		Agave + V-Oct / 9	unknown
206.229		Agave + V-Oct / 9	unknown
224.234		Agave + V-Oct / 9	unknown
289.312	289.158	Agave + V-Oct / 9	$M_{G/F}$ - H_2O + H^+
324.360	324.202	Agave + V-Oct / 9	$M_{G/F}$ + NH_4^+
329.311	329.158	Agave + V-Oct / 9	$M_{G/F}$ + Na^+
415.087	415.269	Honey/Agave unpurified	$M2_{G/F}$ - H_2O + H^+
450.107	450.306	Honey/Agave unpurified	$M2_{G/F}$ + NH_4^+

6.3.4 Analysis of the synthesized products by NMR experiments

Mass spectrometry identified acylated sugars in purified synthesis products of both substrates. To distinguish between glucose- and fructose octanoates, both with an identical calculated molar mass of 306.168 Da, purified and fractionated main components of a synthesis reaction in honey or agave syrup were analyzed by NMR spectroscopy. With honey as substrate, one clear major carbohydrate system was identified in the sample as glucose, starting from the anomeric protons of the ^1H COSY and ^{13}C HMBC spectra. Based on cross peaks of carbohydrate protons with lipid carbonyls in the $^1H^{13}C$ HMBC, the glucose moieties were acylated with octanoic acid at the C6 atoms (Table 6).

The purified synthesis product with agave syrup as substrate clearly revealed a cross peak between the lipid carbonyl C-atom and the CH_2-carbohydrate group in the $^1H^{13}C$ HMBC spectra at 66.1 ppm (^{13}C) and 4.22 and 4.11 ppm (^1H) (Appendix 25). This group shows no further cross peak in the 2D COSY. In the $^1H^{13}C$ HMBC it is connected to a quaternary carbon. This identifies the carbon atoms as C^1H_2 group of fructose, since glucose has no comparable group. A further assignment of the samples resonances was not possible due to spectral overlap with various impurities.

Table 6.4: Chemical shifts of the main product, presented in the purified and fractionated sample of a synthesis reaction of vinyl octanoate in honey with iCalB. Despite purification via flash chromatography, the fractionated sample of the synthesis reaction in agave syrup show many impurities. Based on the identification of acetylated carbon atoms as C^1H_2 group, it is possible to state that it is fructose and not glucose.

glucose	C shift (ppm)	H shift (ppm)
-C^1H-O-	92.53	5.21
-C^2H-	72.27	3.46
-C^3H-	73.73	3.76
-C^4H-	70.03	3.34
-C^5H-	69.64	3.97
-C^6H- (acylated C'174.13)	63.31	4.23
-$C^{6'}$H- (acylated C'174.13)	63.31	4.35

The NMR data confirm the formation of sugar esters in honey and agave syrup. It was determined that the synthesized product in honey, with a ratio of fructose to glucose of 1 to 0.78, is glucose-6-octanoate. This indicates that the used iCalB seems to prefer to acylate glucose at the C6, which was already shown (Siebenhaller et al., 2017a). Whether fructose octanoate is also a product of the synthesis reaction in honey can not conclusively be clarified, as it may have been removed from the measured sample during the purification step. Vice versa, in the purified synthesized product sample in agave syrup, with a molar ratio of one fructose to 0.26 glucose, only acylated fructose was detected. This sample shows various impurities; therefore, further statements should only be taken with caution. Since the yield of di-esters seems very low, NMR analysis was not possible.

The NMR data may indicate that our first presumption was wrong, and the used iCalB prefers glucose to fructose at similar sugar ratios. Whether this is correct or caused by other factors like temperature, fatty acid length or other components in the honey or agave syrup influence the substrate preference, has to be further investigated.

6.4 Conclusion

It was shown that ordinary honey and, as vegan alternative, agave syrup are suitable to act simultaneously as substrates and solvents for the enzymatic synthesis of sugar esters. The successful formation of the sugar esters was proven by TLC, MS and multidimensional NMR experiments, and by comparison to known reactions in sugar-based deep eutectic solvents.

The produced sugar esters can potentially be used in the pharmaceutical or cosmetics industry to improve existing products or to replace conventional surfactants. But to do so, important attributes and dermatological properties have to be determined, first.

Important characteristics like the content of the main sugars, water activity and water content as well as the pH of the herein used honey and agave syrup were determined and compared to literature data. However, this have to be further investigated since they might have an influence on the reaction, yields and products.

The used process is not yet optimized, but it was demonstrated that the reaction system on which this work is based is very versatile and stable. Furthermore, it shows that the used lipase can overcome the used unconventional media and is able to reverse their hydrolytic activity and synthesize sugar esters despite the high water content and water activity. This result may open the door for other natural substrates like maple syrup, rice syrup or the commercially important corn syrup in future.

6.5 References chapter 6

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7 Summary and Conclusion

Surfactants are important molecules with applications ranging from a milligram scale in pharmaceuticals, up to a tons scale as additives in oil drilling. In addition, surfactant like glycolipids have manifold abilities. In this thesis, the main goal was to produce sustainable glycolipids by an eco-friendly enzymatic reaction to change our raw-oil economy into a bio-based society.

In this work, a universal toolbox for the enzymatic synthesis of tailor-made glycolipids was established and all further goals achieved. It is possible to use various sugars and combine them with a broad range of fatty acids in a novel reaction media, so that surfactants with desired properties can be produced for a bio-economy.

The enzymatic synthesis of glycolipids was executed successfully by utilizing sustainable media and substrates. In a first step, a deep eutectic solvent reaction system was established. Sustainable and, depending on the carbohydrate source, environmental friendly DES were formed by utilizing choline chloride and different (anhydro-) sugars or sugar alcohols. Additionally, the sugar component of the DES acts simultaneously as substrate and as part of the reaction media. In all set-ups of DES, enzyme and fatty acid vinyl ester, glycolipids were detected. This indicates that DES are suitable and versatile media for the enzymatic synthesis of tailor-made glycolipids.

In a next step, important synthesis parameters were determined with the sugar ester arabinose laurate as reference in order to optimize the process. It was resolved that the molar ratio of CC and arabinose has only a minor affect on the yields. In contrast, the water content of the DES is a crucial parameter for high reaction yields. By adding water to a concentration of 8 %, the yield of arabinose laurate was doubled to approximately 50 % in 48h. Reasons for this might be a lower viscosity and therefore a better mass transfer or an improved availability of sugar, but further experiments or simulation studies have yet to be done. Despite this, the addition of 12 % water did also decrease the formation of arabinose laurate by a few percent in comparison to DES with only 4 % of water probably to a hydrolytic cleavage of the molecule. The formation of arabinose di-laurates was also influenced by the water content. When adding water, no di-laurates were synthesized. However, water might be a promising additive for an industrial glycolipid production in the applied system.

Whether and in which fields glycolipids will be used depends not only on their biocompatibility, biodegradability or their production cost, but also strongly on their properties. Therefore, the surface properties of pure arabinose laurate were determined. The results show that it is able to reduce the surface tension of water to air drastically to 23 mN/m. The CMC value with 530 mg/L is quite high in comparison to other known glycolipids, but it could be an interesting surfactant for water-based application due to its relatively short acyl chain. Further properties such as bubble size or foam stability, as well as carbonyl chain length and saturation state are investigated in a follow-up project.

The possibility of using a DES consisting of CC and a carbohydrate for glycolipid synthesis was demonstrated. It has been investigated whether sugar from lignocellulose, the most common renewable raw material in the world, can be used as a substrate. For this, a monosaccharide solution was purified, dried and used as component for DES formation. In this sustainable media, the successful formation of glucose and xylose esters were verified and a yield of 4.8 % was reached. The further aim of this work was to produce glycolipids entirely from renewable raw materials, which are not competing with food or feed industry. For this reason, the untreated beech wood cellulose fiber hydrolysate was used as sole carbon source for the yeast *Cryptococcus curvatus* to produce single cell oil by fermentation. This SCO has a fatty acid profile similar to many plant oils, with 44.6 % oleic acid (C18:1), 35.1 % palmitic acid (C16:0) and 12.6 % stearic acid (C18:0) as major fatty acids. The SCO production with this substrate was comparable to other C-sources, like pure sugars, and could replace these higher-grade substrates. The synthesis reaction with iCalB as enzyme with the produced fatty acids leads to a glycolipid mixture. Several sugar esters like glucose palmitate, glucose oleate or di-esters with palmitic acid and stearic acid or two linoleic acids were detected. Some rarer xylose esters were identified, too.

Additionally, it was possible to simultaneously use honey and agave syrup as media and substrate to synthesize glycolipids. From a bioeconomical perspective, these media should not be favored and only be used for special applications in cosmetic or wound treating.

In further projects, it has to be investigated how the fatty acid chain length, the saturation state and kind of carbohydrate influence the surface activity, as well as how the reaction can be up scaled and optimized.

To sum up, the utilization of three renewable raw materials has been successfully demonstrated. The usage of honey and agave syrup could be very interesting for cosmetics or pharmaceutical applications, but from a bio-economic point of view, they are less suitable for large scale production since they are used mainly in the food industry. Lignocellulose is a very promising resource and can be found in millions of tons as waste or as a cheap side product. In the presented integrated process, lignocellulose can be easily used as a sustainable resource for the production of 100% bio based glycolipids.

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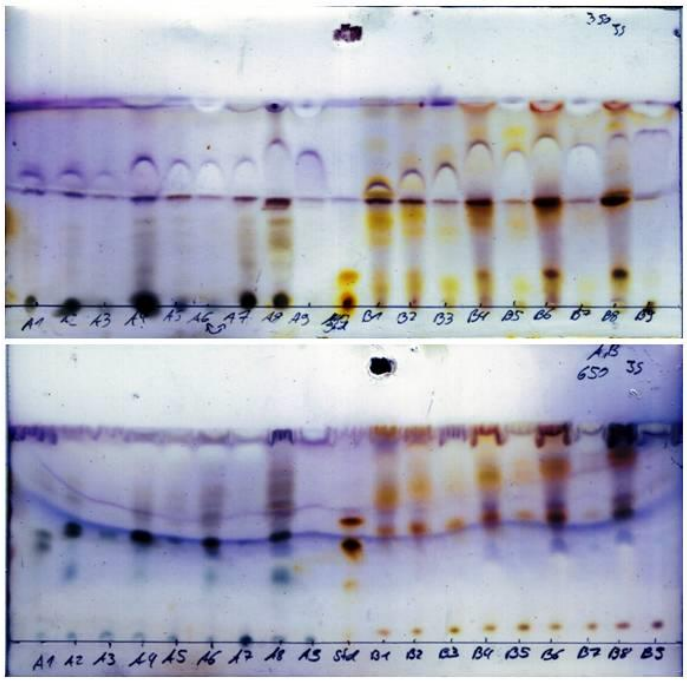
Appendix

Appendix 1: TLC of synthesis products in a DES of CC:Glucose



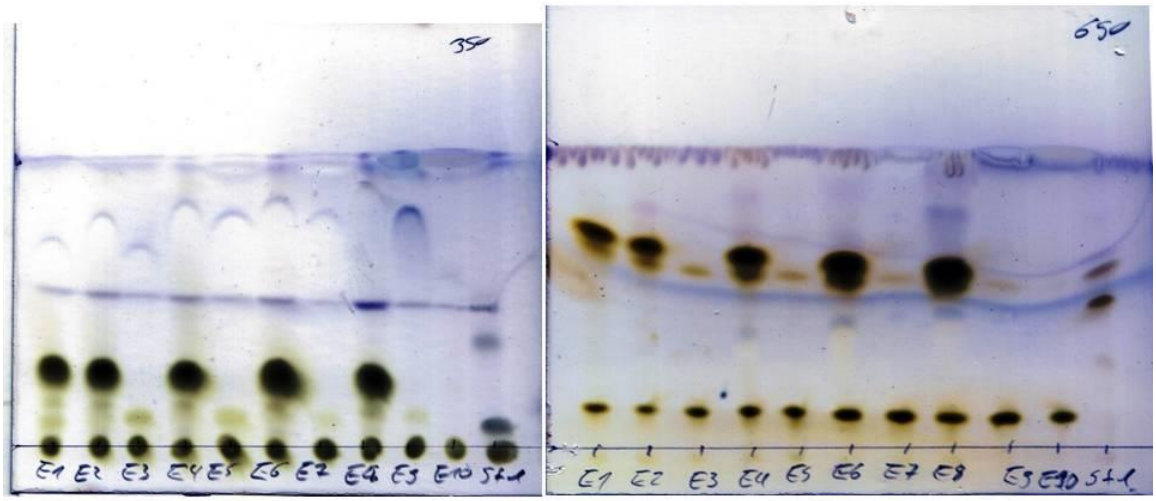
A = a DES, consisting of choline chloride and glucose
B = a DES, consisting of choline chloride and xylose

1 = Vinyl hexaonate; 2 = Vinyl octanoate; 3 = Methyl octanoate; 4 = Vinyl decanoate; 5 = Methyl decanoate; 6 = Vinyl laurate; 7 = Methyl laurate; 8 = Vinyl palmitate; 9 = Methyl palmitate;
10 = DES with vinyl laurate without enzyme as negative controlle

Std = Labintern standard

Above: mobile phase chloroform : methanol : acetic acid (35 : 1 : 1, by vol.)
Below: mobile phase chloroform : methanol : acetic acid (65 : 15 : 2, by vol.)

Appendix 2: TLC of synthesis products in a DES of CC:Rhamnose.

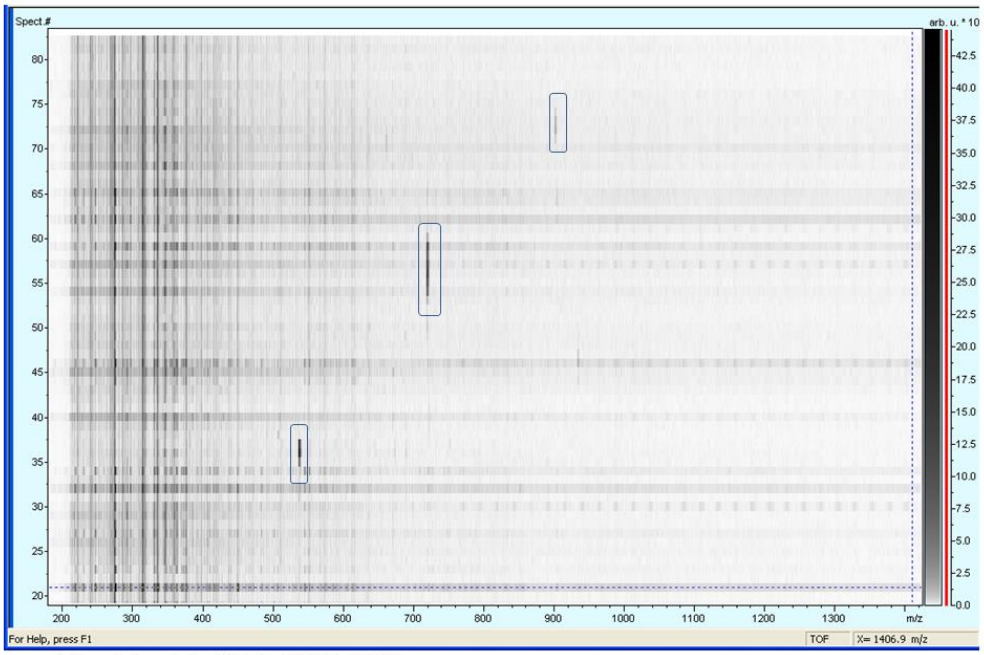


E = a DES, consisting of choline chloride and Rhamnose
1 = Vinyl hexaonate; 2 = Vinyl octanoate; 3 = Methyl octanoate; 4 = Vinyl decanoate; 5 = Methyl decanoate; 6 = Vinyl laurate; 7 = Methyl laurate; 8 = Vinyl palmitate; 9 = Methyl palmitate; 10 = DES with vinyl laurate without enzyme as negative controlle
Std = Labintern standard

Left: mobile phase chloroform : methanol : acetic acid (35 : 1 : 1, by vol.)
Right: mobile phase chloroform : methanol : acetic acid (65 : 15 : 2, by vol.)

Appendix 3: TLC-MALDI-ToF of arabinose laurate.

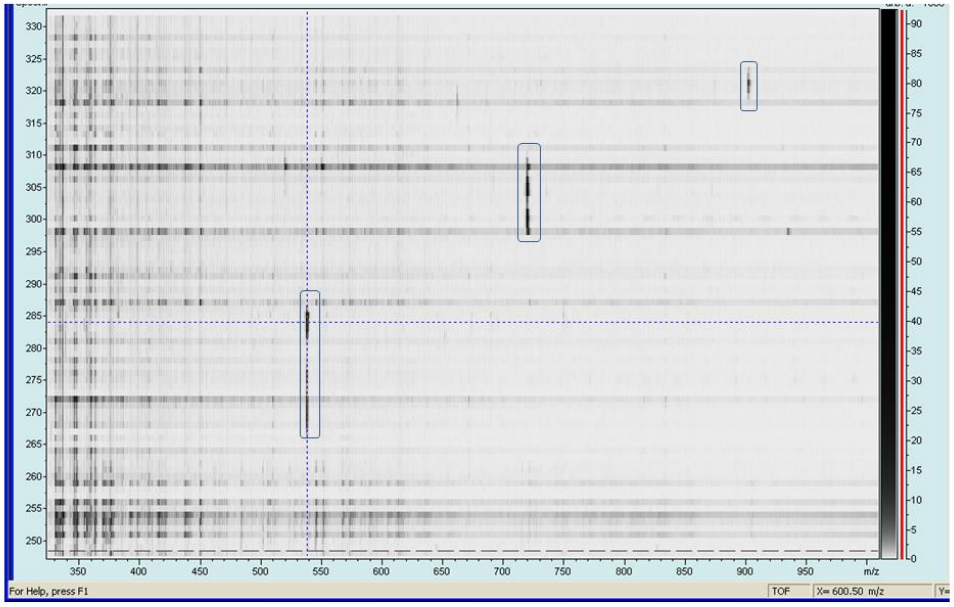
Arabinose-laurate



m/z from left to right: 538.02; 719.57; 901.48

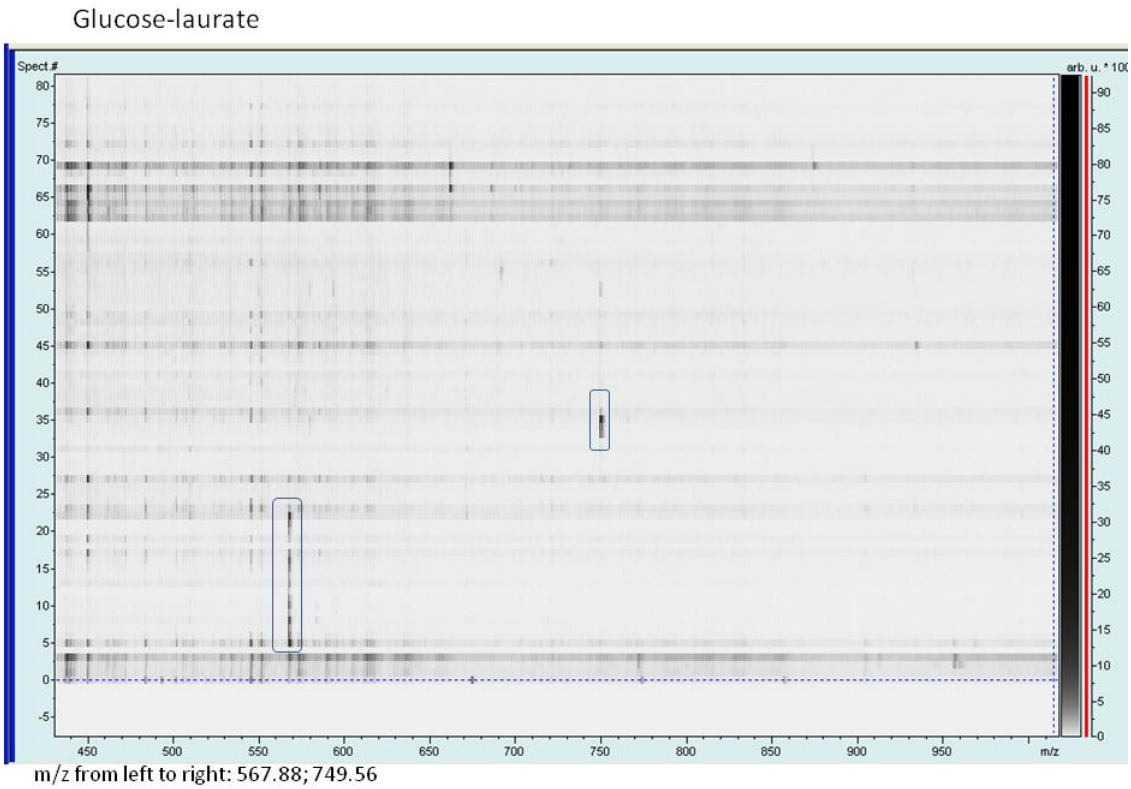
Appendix 4: TLC-MALDI-ToF of xylose laurate.

Xylose-laurate



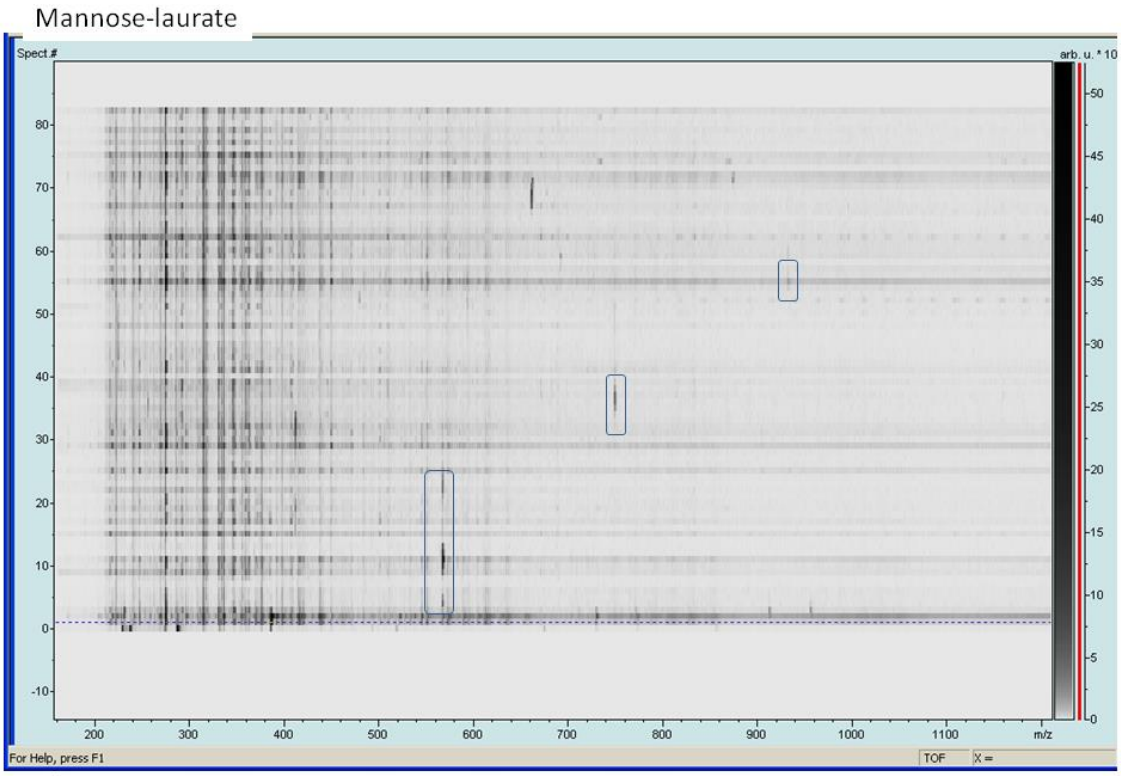
m/z from left to right: 537.97; 719.37; 901.42

Appendix 5: TLC-MALDI-ToF of glucose laurate.



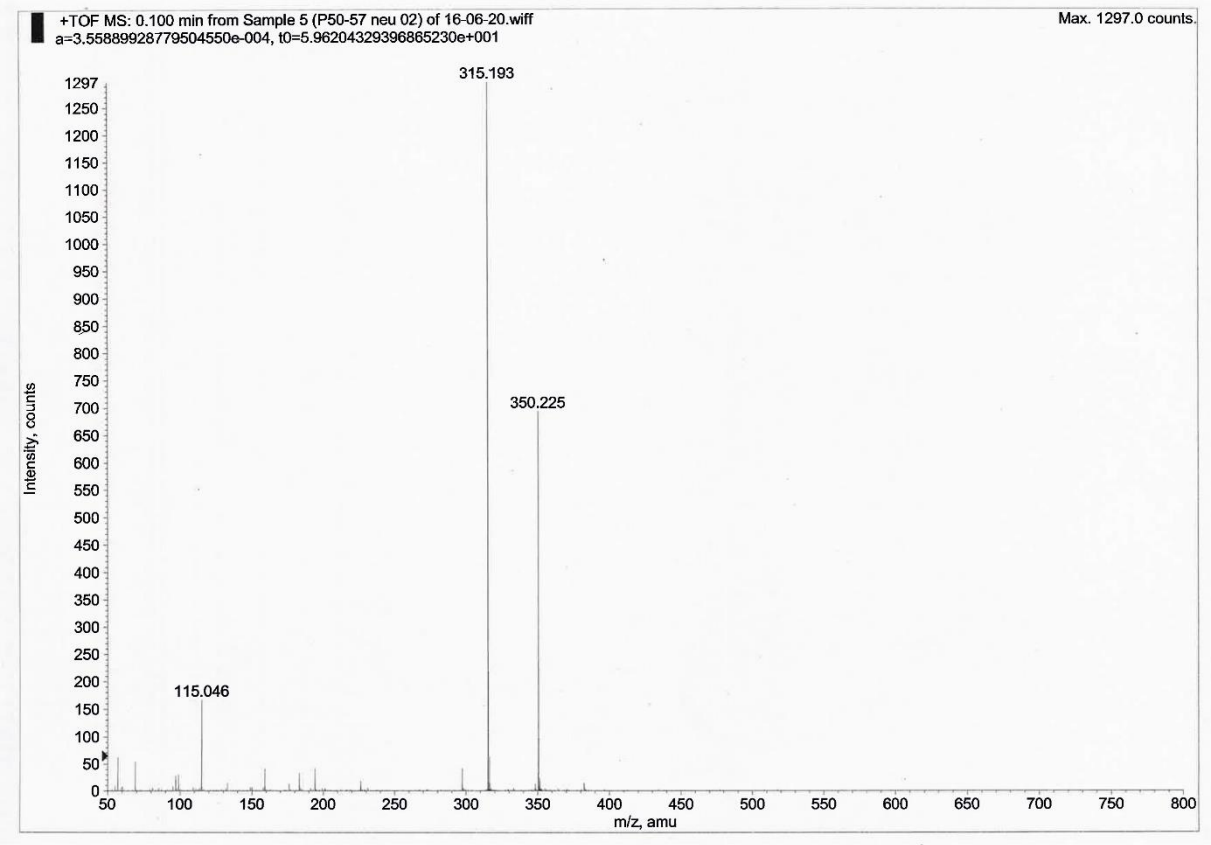
m/z from left to right: 567.88; 749.56

Appendix 6: TLC-MALDI-ToF of mannose laurate.

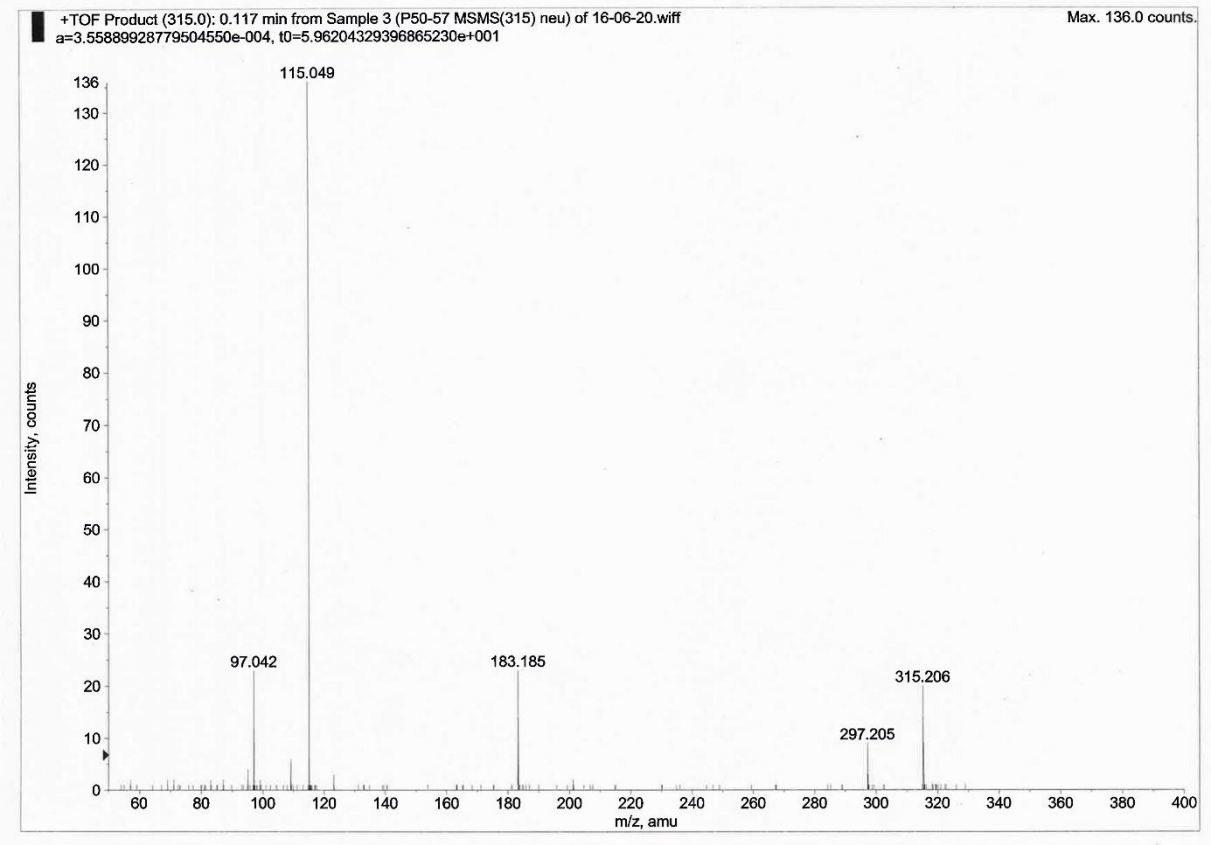


m/z from left to right: 567.92; 749.42; 931.44

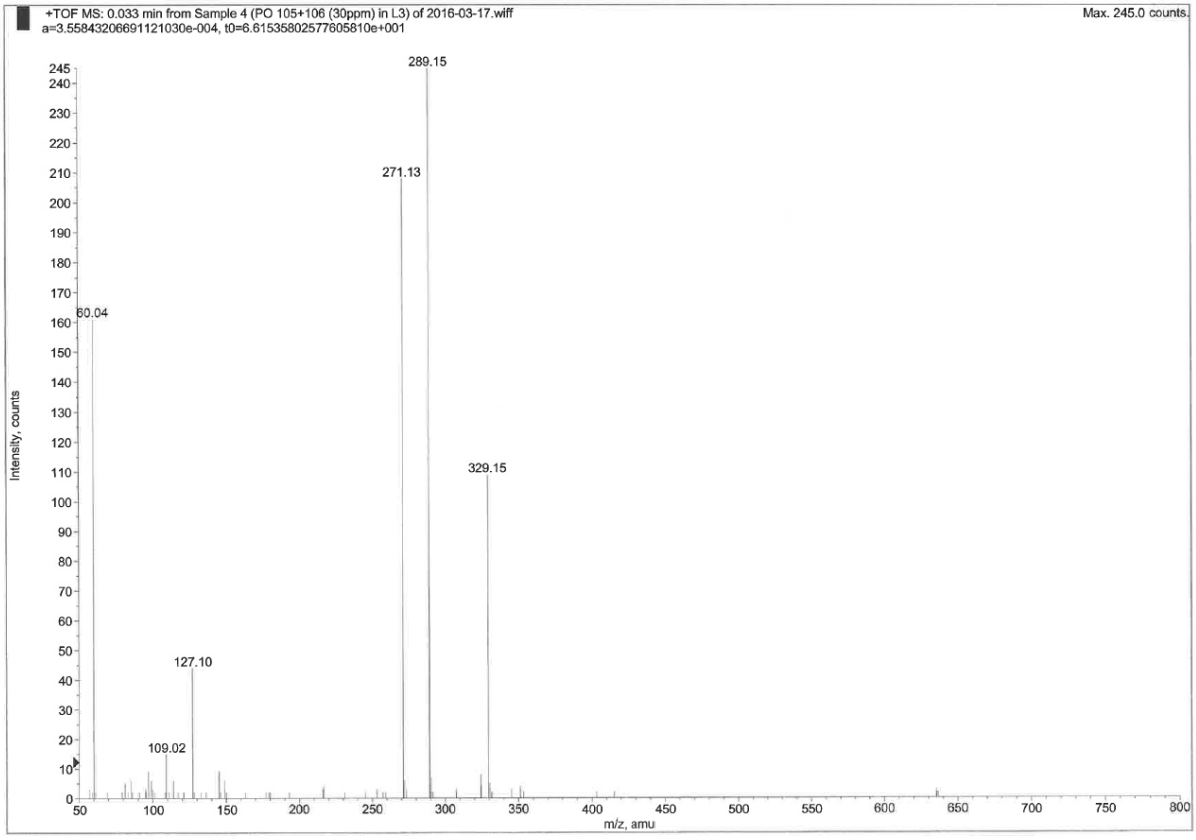
Appendix 7: ESI-ToF of purified fraction 50-57 for confirmation of arabinose laurate.



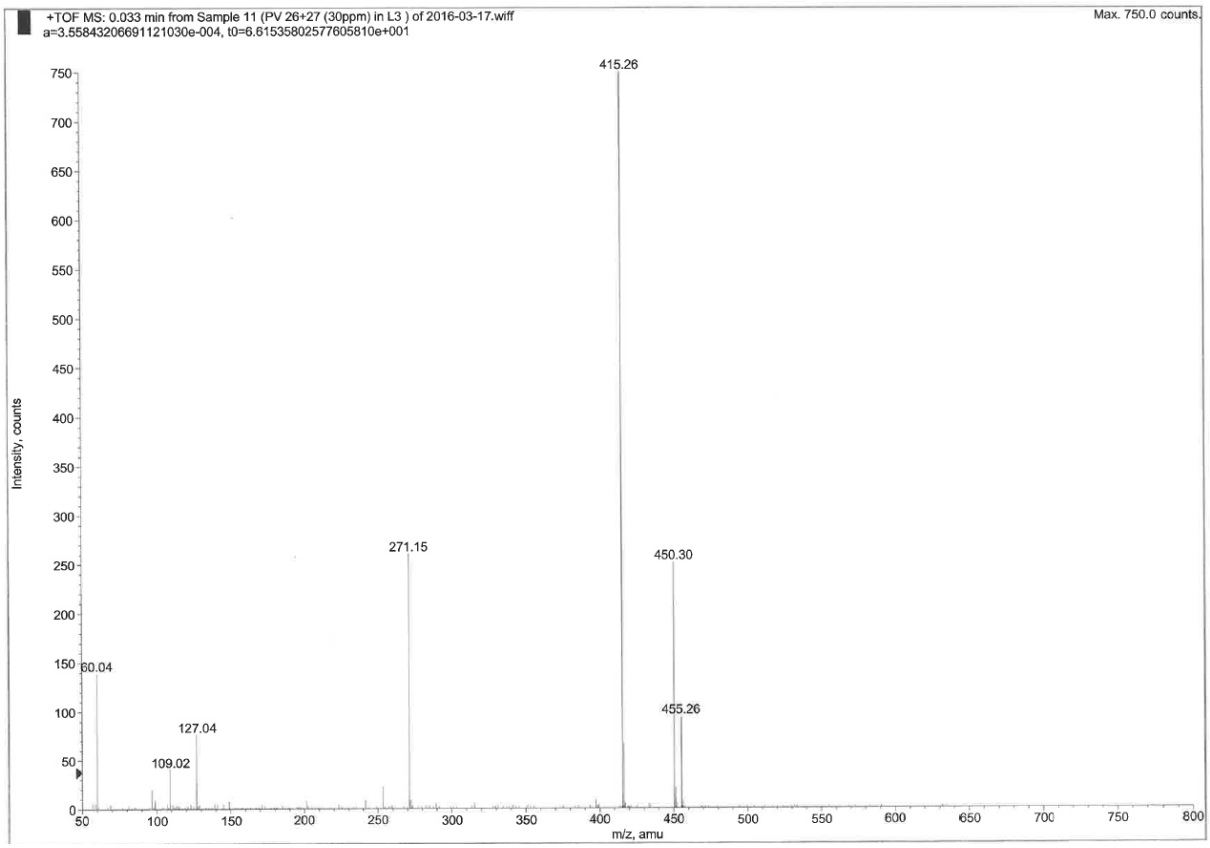
Appendix 8: MS/MS of synthesized arabinose laurate.



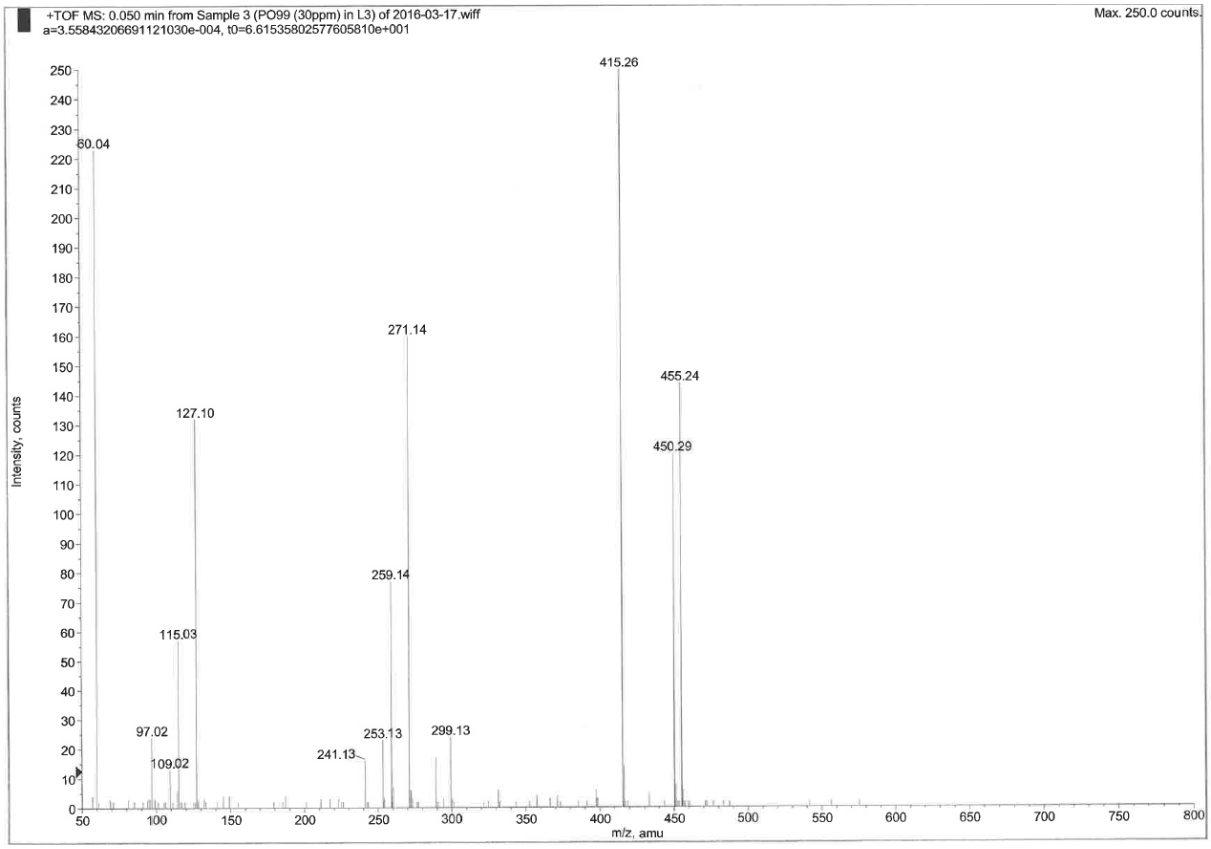
Appendix 9: ESI-Q-ToF of synthesized glucose octanoate with glucose from beech wood.



Appendix 10: ESI-Q-ToF of synthesized glucose di-octanoate with glucose from beech wood.



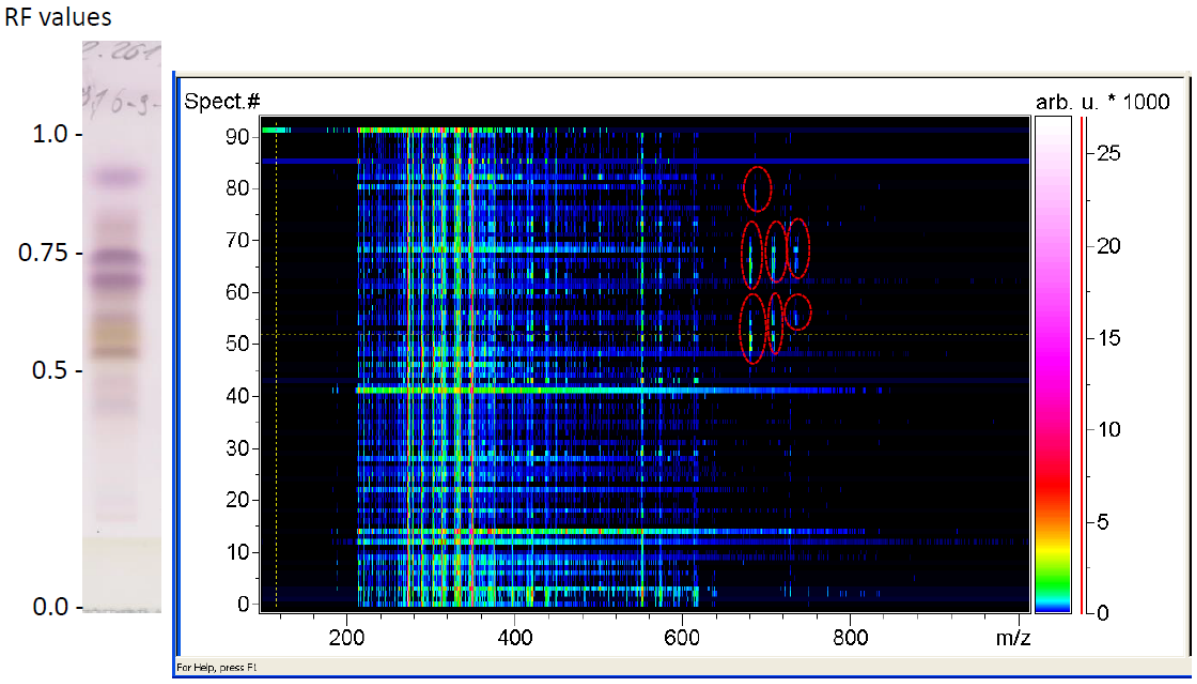
Appendix 11: ESI-Q-ToF of synthesized glucose di-octanoate with glucose from beech wood.



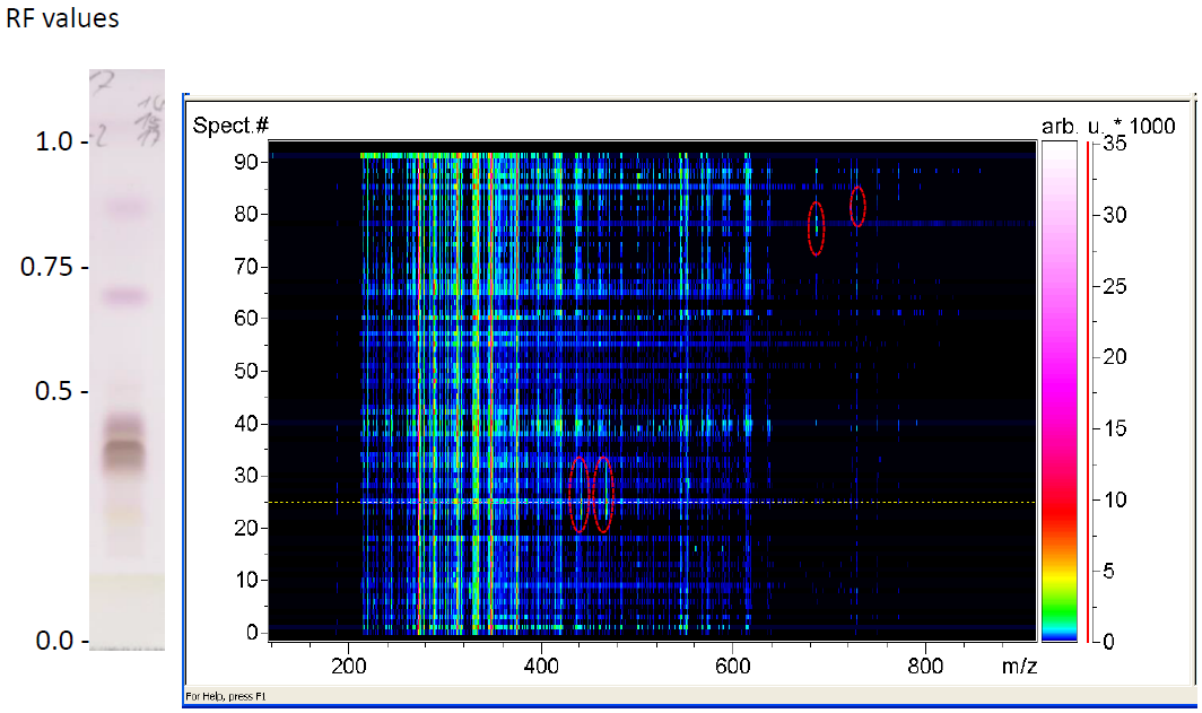
Appendix 12: All measured data from the fermentation with mean values and standard deviation.

	Carbon source (g/L)				Dry weight (g/L)				Ammonium (g/L)		Lipids (% lipid/BDM)	
Time (h)	Batch 1	Batch 2	Mean	Standard deviation	Batch 1	Batch 2	Mean	Standard deviation	Batch 1	Batch 2	Batch 1	Batch 2
0.0	36.36	37.52	36.94	0.82	0.3	0.5	0.4	0.1	1.38	1.41		
1.9	46.59	44.48	45.54	1.49	0.3	0.6	0.5	0.2				
3.9	43.85	40.37	42.11	2.46	0.6	0.9	0.8	0.2				
6.0	42.57	44.48	43.53	1.35	1.3	1.1	1.2	0.1	1.16	1.24		
23.9	0.06	0.41	0.24	0.25	22.2	23.4	22.8	0.8	0.00	0.00		
25.9	0.06	0.35	0.21	0.21	22.2	23.4	22.8	0.8				
27.8	0.02	0.31	0.17	0.21	22.3	23.1	22.7	0.6				
29.9	48.35	48.99	48.67	0.45	20.8	21.2	21.0	0.3				
47.8	0.06	0.09	0.08	0.02	34.1	34.7	34.4	0.4			11.89	12.69
49.8	0.06	0.04	0.05	0.01	33.9	33.6	33.8	0.2				
51.8	0.08	0.04	0.06	0.03	34.3	35.1	34.8	0.6				
53.8	47.32	51.53	49.43	2.98	28.3	27.2	27.8	0.8			38.62	32.17
71.8	0.08	0.30	0.19	0.16	45.6	44.3	45.0	0.9				
73.8	0.09	0.20	0.15	0.08	43.7	44.3	44.0	0.4				
75.7	0.11	0.19	0.15	0.06	43.3	41.2	42.3	1.5				
77.8	55.68	47.92	51.80	5.49	30.6	31.4	31.0	0.6			42.02	47.12
95.8	1.31	0.25	0.78	0.75	29.4	33.5	31.5	2.8			47.60	48.08

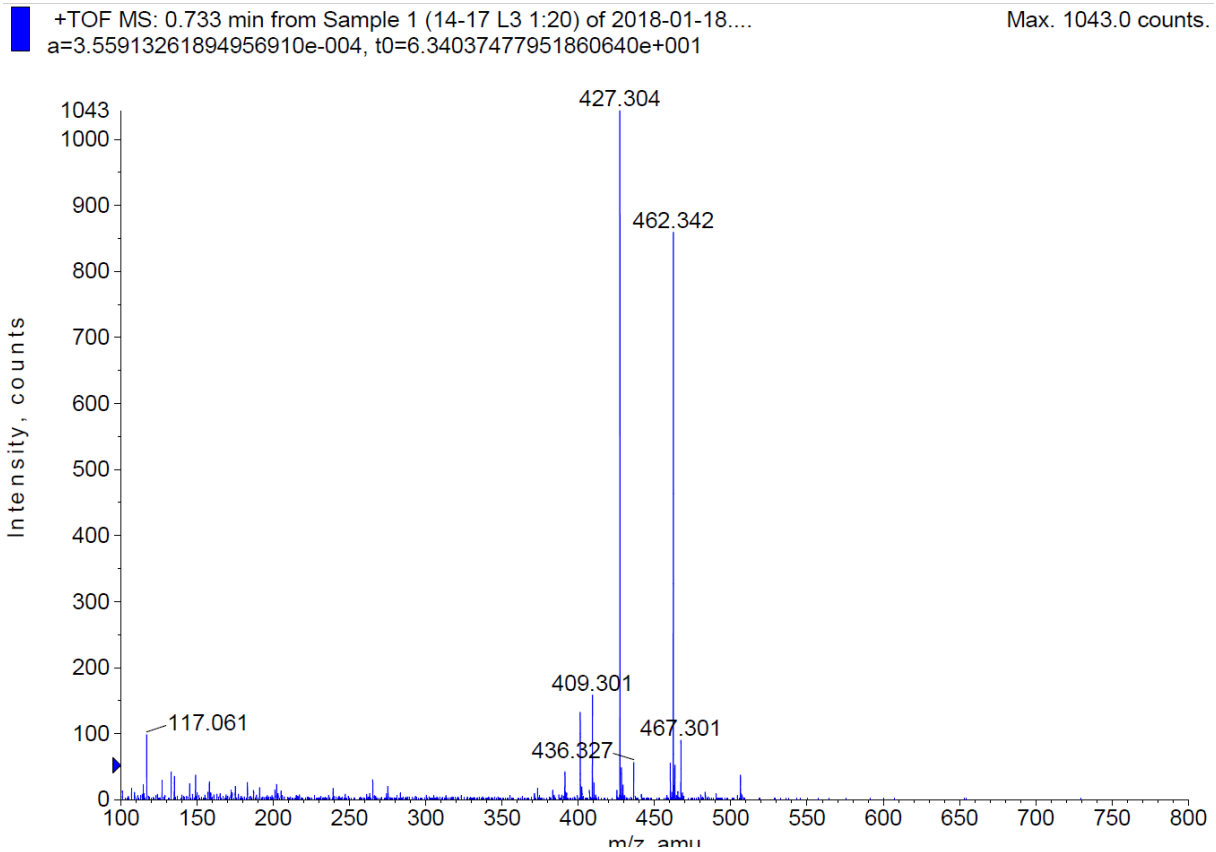
Appendix 13: TLC-MALDI-ToF MS of fraction 6-9 from purified synthesis products completely made from beech wood.



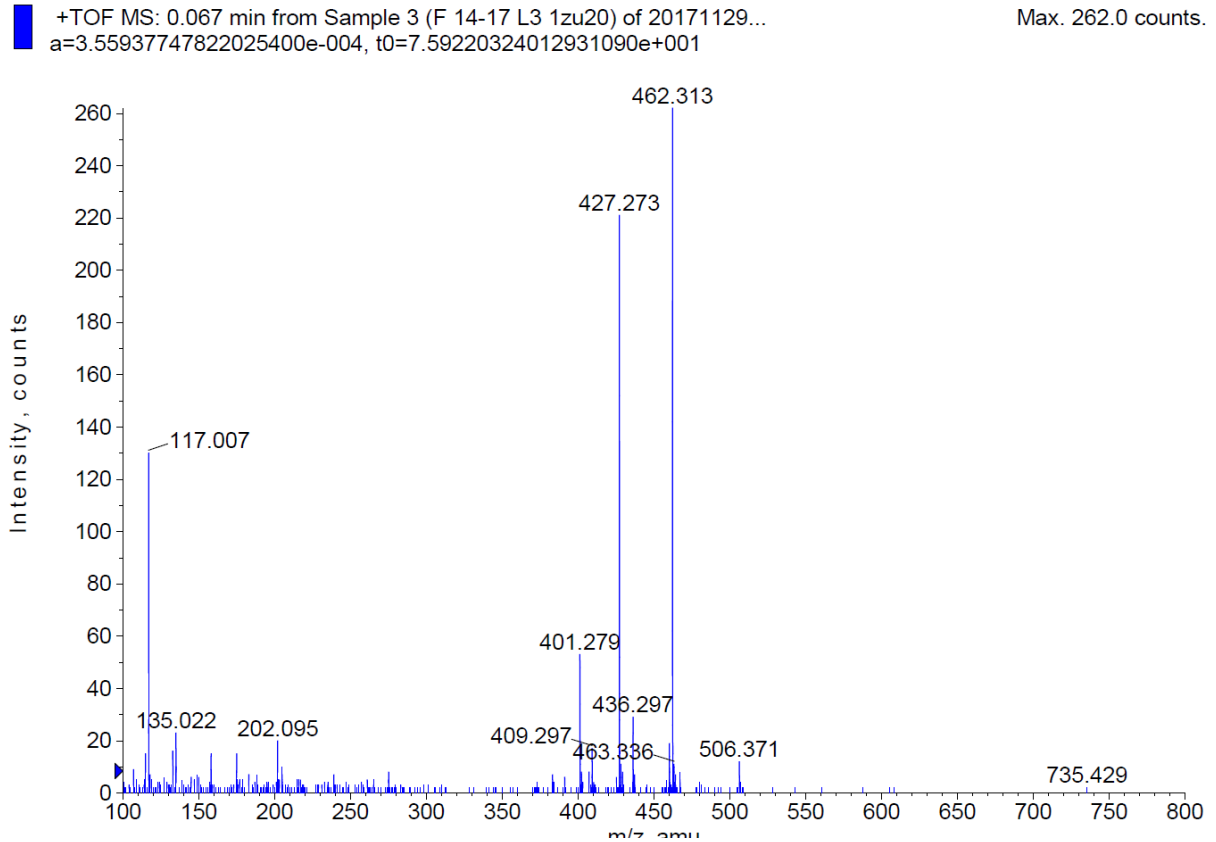
Appendix 14: TLC-MALDI-ToF MS of fraction 14,15,17 from purified synthesis products completely made from beech wood.



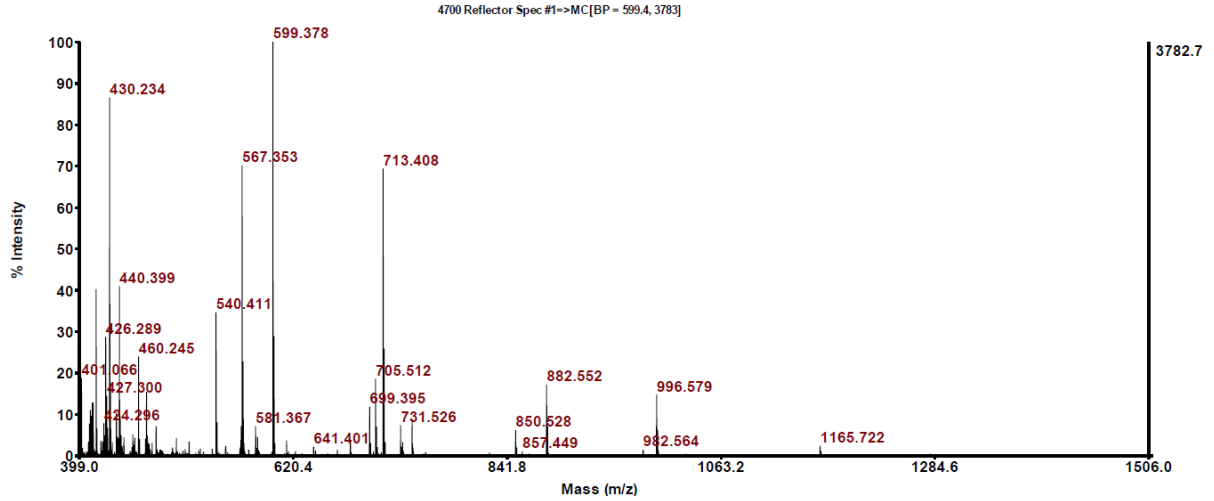
Appendix 15: ESI-Q-ToF from glycolipids which are based completely from beech wood.



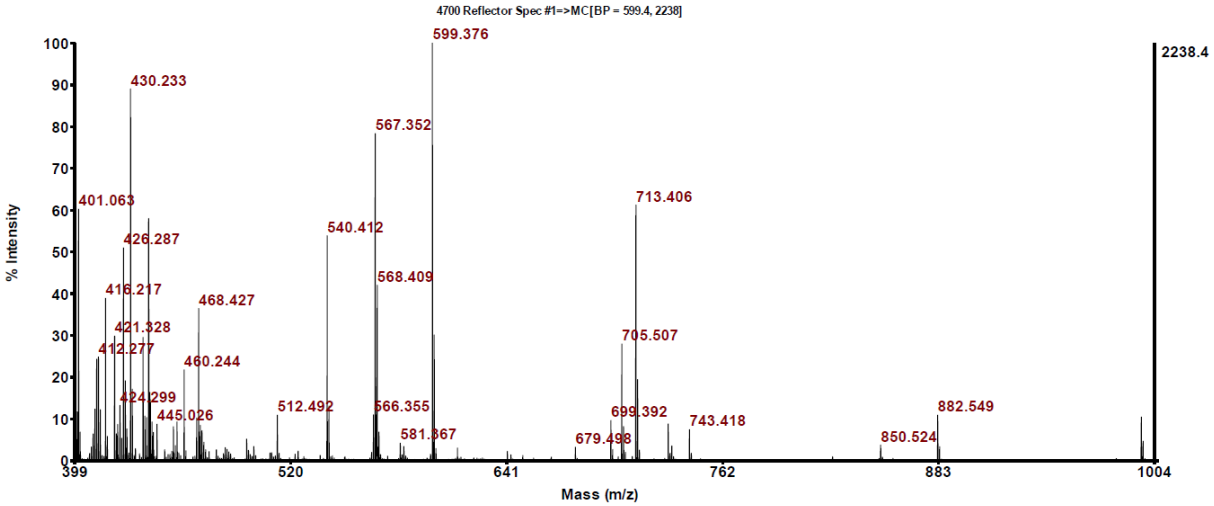
Appendix 16: ESI-Q-ToF from glycolipids which are based completely from beech wood.



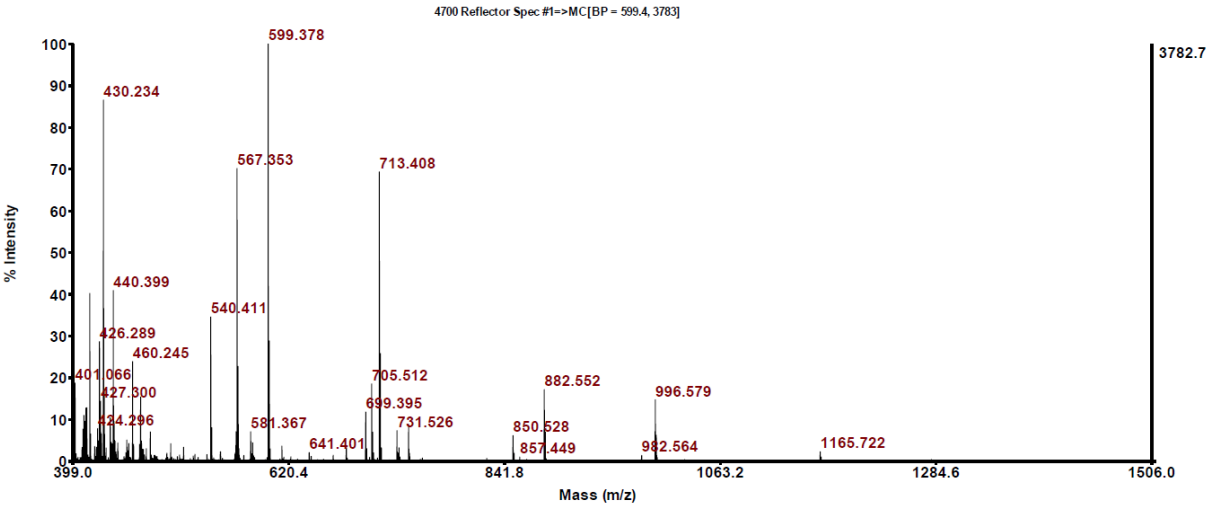
Appendix 17: MALDI-ToF of purified glycolipids in sample 6-9.



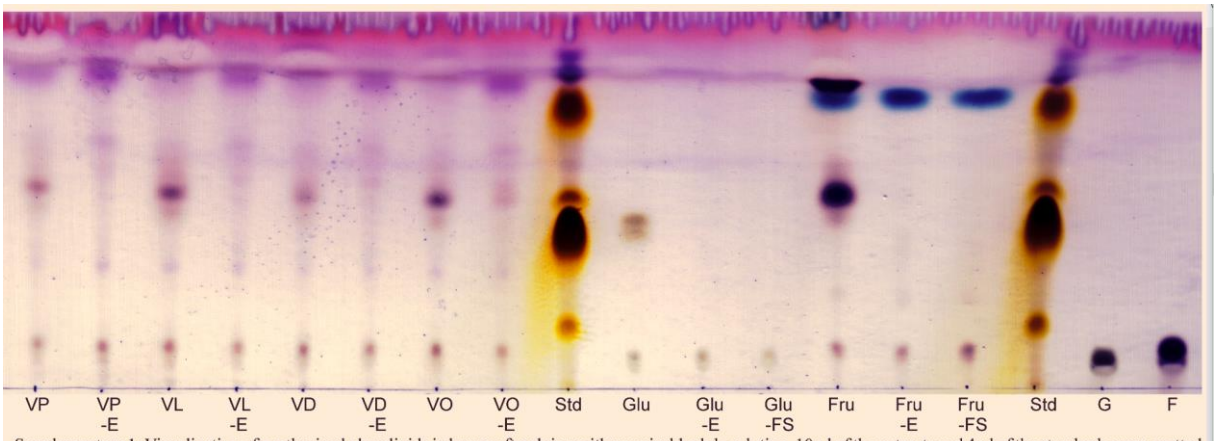
Appendix 18: MALDI-ToF of purified glycolipids in sample 6-9.



Appendix 19: MALDI-ToF of purified glycolipids in sample 6-9.

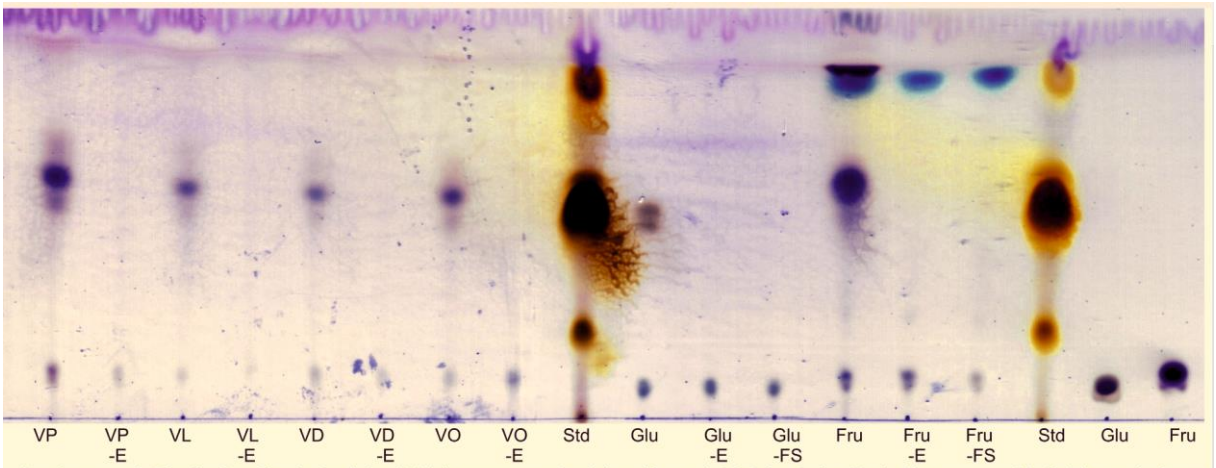


Appendix 20



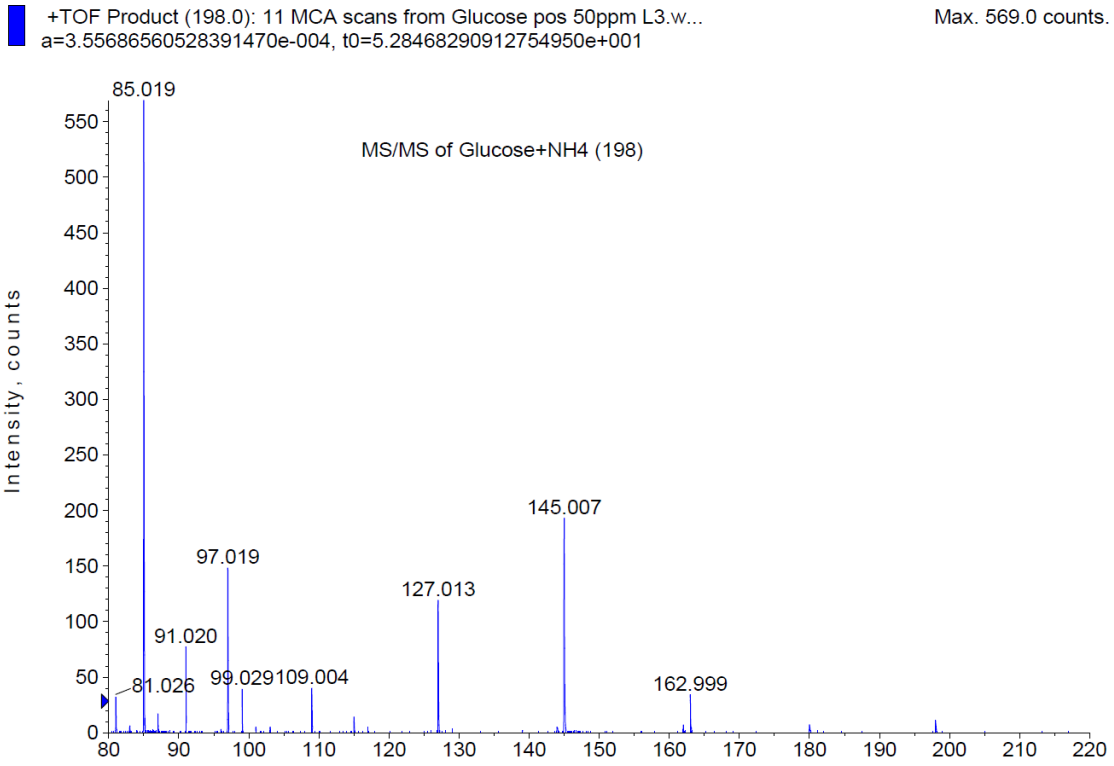
Supplementary 1: Visualization of synthesized glycolipids in honey after dying with an anisaldeyhde solution. 10 µl of the extracts and 4 µl of the standards were spotted on the TLC plate. VP = vinyl-palmitate; VL = vinyl-laurate; VD = vinyl-decanoate; VO = vinyl-octanoate; Std = lab intern rhamnolipid standard; Glu = glucose based DES with VO; Fru = fructose based DES with VO; G = glucose, solved in a ethanol-water mixture; F = fructose in a ethanol-water mixture.

Appendix 21

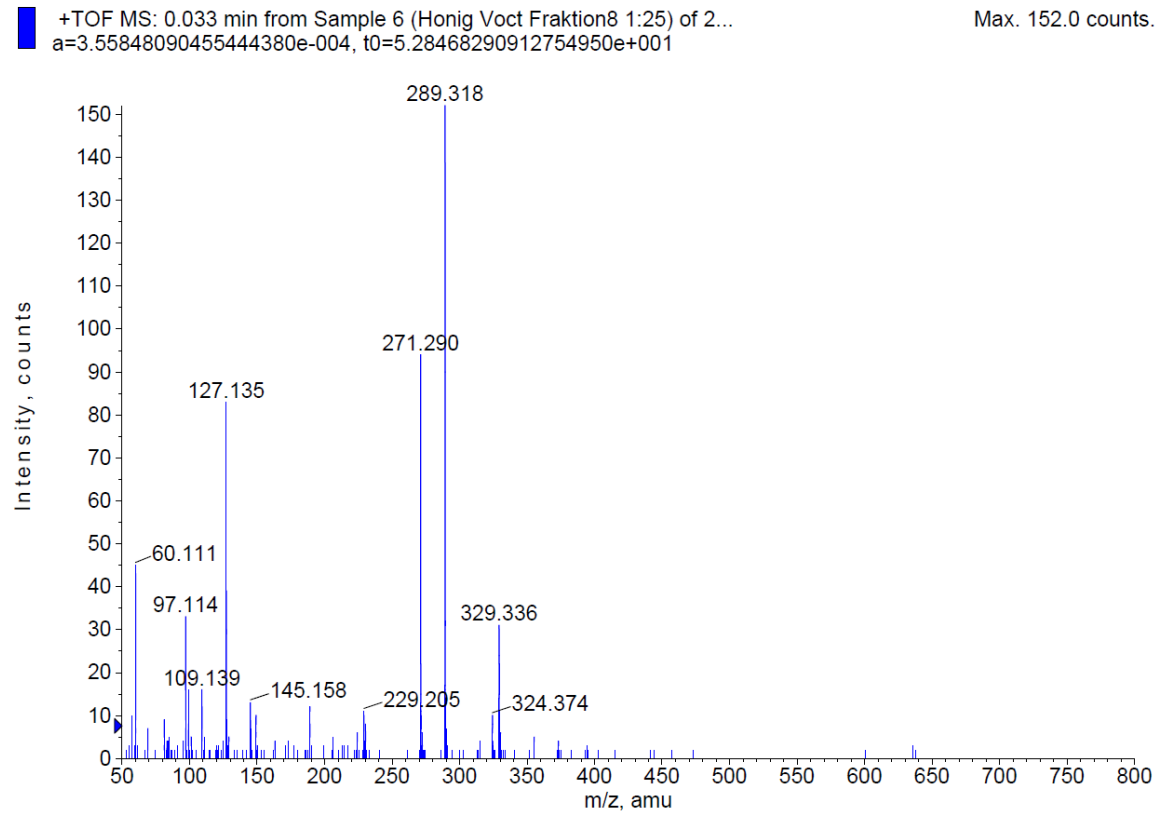


Supplementary 2: Visualization of synthesized glycolipids in agave syrup after dying with an anisaldeyhde solution. 10 µl of the extracts and 4 µl of the standards were spotted on the TLC plate. VP = vinyl-palmitate; VL = vinyl-laurate; VD = vinyl-decanoate; VO = vinyl-octanoate; Std = lab intern rhamnolipid standard; Glu = glucose based DES with VO; Fru = fructose based DES with VO; G = glucose, solved in a ethanol-water mixture; F = fructose in a ethanol-water mixture.

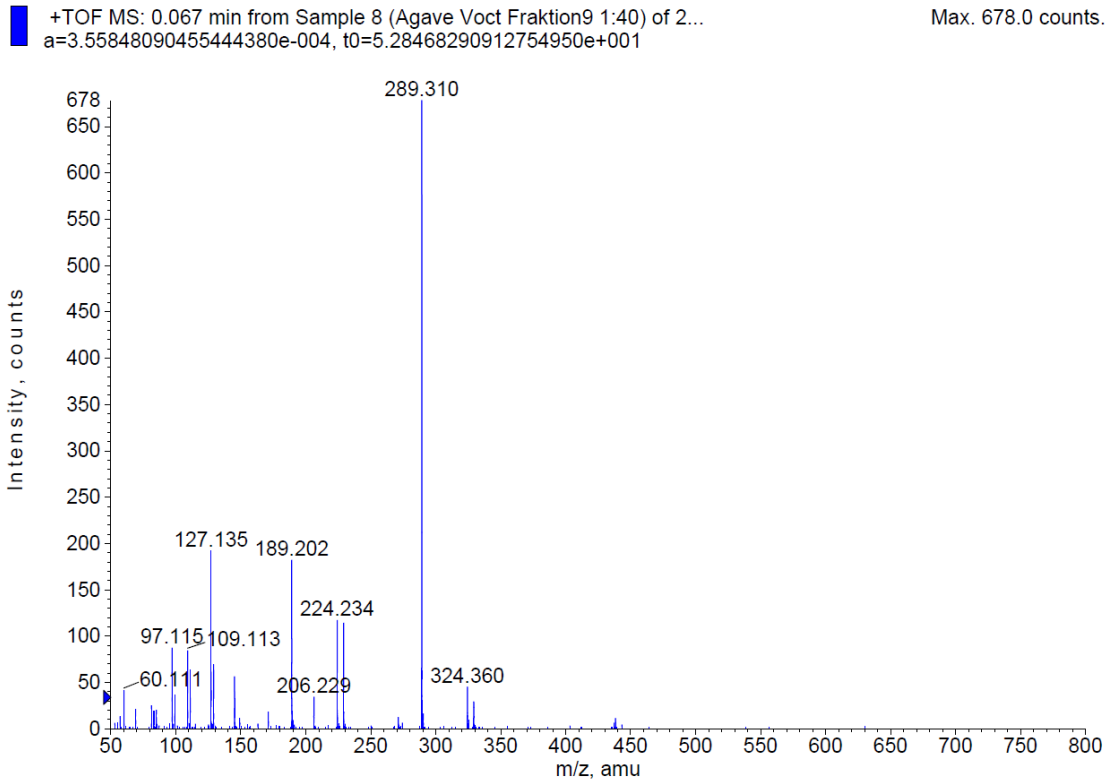
Appendix 22: Verification of sugar cleavage products



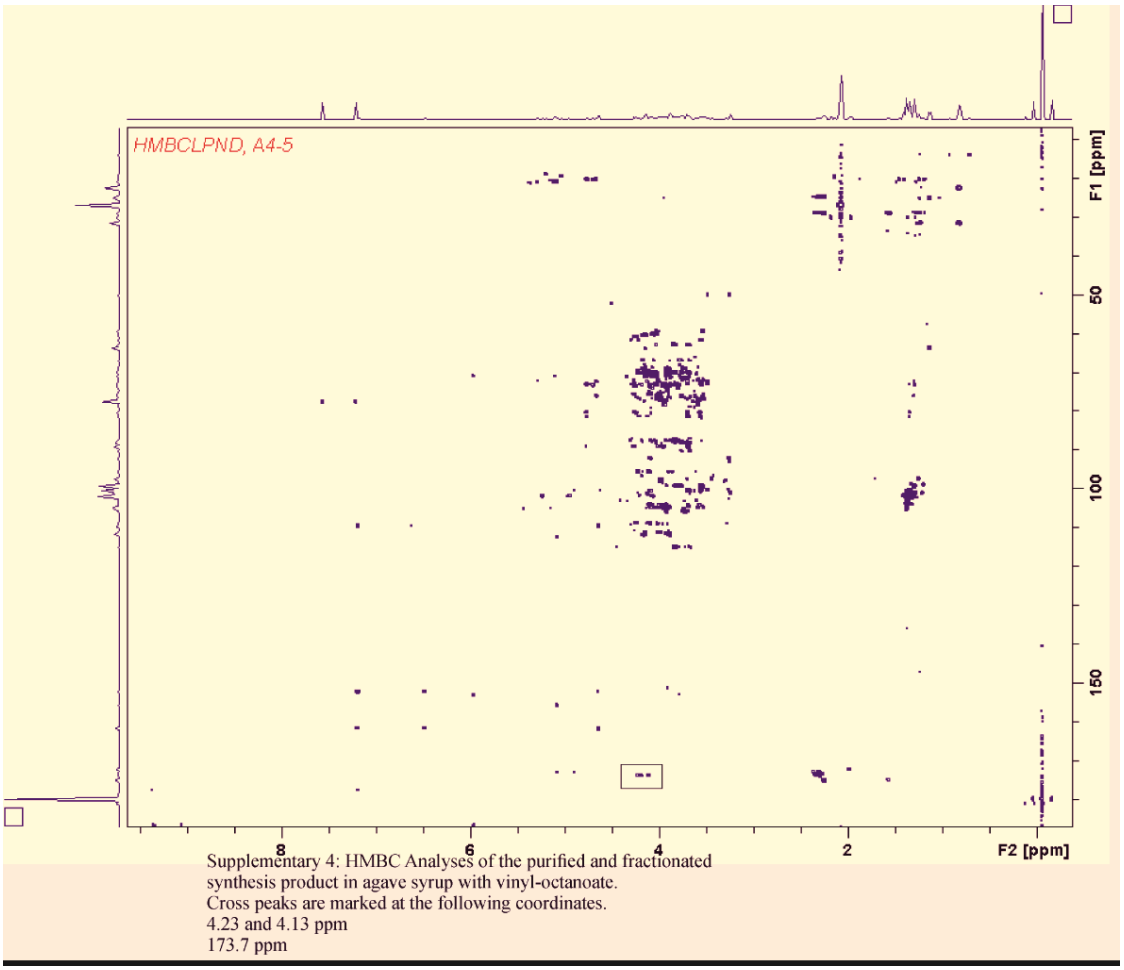
Appendix 23: Synthesis with honey and vinyl octanoate, fraction 8



Appendix 24: Synthesis with agave syrup and vinyl octanoate, fraction 9



Appendix 25: HMBC-NMR of glycolipids



Supplementary 4: HMBC Analyses of the purified and fractionated synthesis product in agave syrup with vinyl-octanoate.
Cross peaks are marked at the following coordinates.
4.23 and 4.13 ppm
173.7 ppm