Lipase-catalysed synthesis of glycolipids
- utilization of innovative media, reactors and substrates -

Zur Erlangung des akademischen Grades eines

DOKTORS DER INGENIEURWISSENSCHAFTEN

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

DISSERTATION

von

M. Sc. André Delavault

aus Aurillac, Frankreich

Tag der mündlichen Prüfung: 17.08.2021

Erstgutachter: Prof. Dr. Christoph Syldatk

Zweitgutachter: Prof. Dr. Matthias Franzreb

Difficulties strengthen the mind,
as labour does the body.
(Lucius Annaeus Seneca)

Acknowledgements

This work is a proof of my determination and perseverance. I dedicate it, nonetheless, to all the persons, family, friends, or colleagues that have supported me and made this success possible, from near or far, for better or worse, through thick and thin, during not only my time as a PhD student, but also throughout my entire curriculum.

To my grandpa, Georges Delavault, and my two uncles Albert and Jean-Pierre Lardies, that have shown me what "Fortitude" means. This work is for you.
It marks the "beginning of the end" of my scientific career, I shall thus forever keep these words as a personal reminder for the storms and calms to come.

"Struggle, challenge, and rise to struggle again."

I would like to thank my supervisor Prof. Dr. Christoph Syldatk for the opportunity he gave me to elevate myself, as a scientist but first and foremost as a Man. His kindness, guidance, and sincere trust are models of exemplarity for what a good leader/mentor should be.

I express my deepest gratitude to PD Dr. Katrin Ochsenreither who showed me an unweathering support for all my decision making, reviewing, and endeavours, whenever I needed it. She motivated me and gave me some "tough-love" when I entailed it the most.

To all my past and present TeBi-Family members. It was such a great time working with all of you that made this relaxed, but nevertheless productive atmosphere at the institute. Here are my special mentions:

- Sascha, my dear friend for bringing me to Germany as a "Frenchy pup′". Your home was always opened to me and you took care of me like your own family with Katja. I love you both so much.
- Teresa, my German sister for guiding my first steps in this new country and never failed to invite me home, so I could get to socialize and not stay alone in my room in the beginning.
- Marcus and Carolin, my colleagues/neighbours for these good moments at the Lessingstr. 40.
- Olga, a.k.a "Olgininski" for all our good talks and her example as a good scientist.
- Rebecca, my Doktor-Schwester whose productivity, intelligence and organization were truly an inspiring model for me.
- Delphine, my friend, and partner in crime for all these microorganisms we have terminated together with our special honey pastures. Thank you for introducing me to the basics of cell-culture.
- Pascal (and all the technician team), a.k.a "Pascalo" for all our laughs, silly jokes, and funny talks together. My "HPLC-Hero".
- My dear Beate and Susanne for the help and nice talks around a coffee at 7:00 am.
- Jens, a.k.a "Grüni" for our good memories of conferences together across Germany and Europe.
- Habib, a.k.a "Sir" for our debates and philosophical talks around a beer.
- Aline and Christin for supporting my laments in Room 104 when things were tough!

Also: Alba, Stefan and Olli for all our good laughs and memories of the "Old School" TeBi!

I´d like to thank my scientific co-workers/friends outside the TeBi that helped me achieving directly or indirectly this work, notably:

- From Montpellier: my Big Brother Erwann Durand and Pierre Villeneuve for inspiring me to do a PhD, they knew how to steer me in the right direction in moments of doubt back when I was a Master student. Also, Thierry Durand and Jérôme Lecompte´s precious words that I "have what it takes to do a PhD".
- From Karlsruhe: Karolin Kohnle, Jasmin Busch and Gregor Klein from the IOC who assisted me by performing experiments that directly helped this work. Hannah Soergel and Dr. Claudia Muhle-Goll from the Campus Nord for their immediate assistance for NMR measurements alongside Kersten Rabe and Ahmed Zoheir for the nice collaboration concerning biosensing.
- From Stuttgart: Fabian Haitz and Susanne Zibek for always offering to help me fulfill the sampling plans of the project.
- Across Germany and Europe: All industrial and academic partners of the Allianz Biotenside and SurfGlyco projects. Isabel Thier and Karsten Siems (Analyticon) for MS and NMR experiments, Bernd Seidendorf (Macherey-Nagel) for purification advice, Ute Wagner (Anton-Paar) for microwave reactor advice, Ramiro Guttierez Martinez (Novozymes) and Andreas Vogel (c-Lecta) for the gracious gifts of enzymes.

I am grateful for the financial support by the *Bundesministerium für Bildung und Forschung* (BMBF) and the diligent work of my dear students and HiWis: Liyin Cai, Deniz Ates, Katarina Ochs, Oleksandra Opochenska and Laura Laneque.

I´d like to thank my father Gilles, my mother Huguette, my sister Gaëlle, my brother-in-law Mathieu, Christine and my grandma Marie-Antoinette for their continuous support and encouragement despite the geographical/temporal distance between us.

Thanks also to my Brothers in Iron from the ASV 04 Grötzingen for the heavy workouts and competitions together: Marvin, Vincent, Walton, Philip and Georg for welcoming me nicely.

My dear Maddi, I owe you so much for the support you brought me. You knew how to rekindle the fire of my sheer will when times were tough mentally and physically. I feel indefinitely lucky that I had a partner like you to endure the challenges we have been through. Your arms were always open to me when I was feeling "too small" for my task, and when I felt like Life was trying to wolf me down. You bring the best out of me, I love you.

Preamble

Parts of this thesis are based on peer-reviewed research articles. All articles have been drafted during this work and describe the major findings of the investigation and optimization of glycolipid synthesis in unconventional media using innovative reactor systems. Chapters which have been previously published or redacted as drafts are indicated as such at the beginning of the chapter. The text of these chapters is partly identical to the publication. Layout, citation style and figures have been modified to match the formatting of this thesis.

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

Enzymatic Glycolipid Surfactant Synthesis from Renewables
Jens Grüninger*, André Delavault*, Katrin Ochsenreither
Process Biochemistry, 2019, 87, 45–54. doi: 10.1016/j.procbio.2019.09.023
*Co-first authorship

Chapter 2 demonstrates the optimization of sorbitol laurate synthesis in a "2-in-1" deep eutectic system and the scalability of the process. This chapter is based on the publication:

Lipase-Catalyzed Production of Sorbitol Laurate in a "2-in-1" Deep Eutectic System: Factors Affecting the Synthesis and Scalability
André Delavault, Oleksandra Opochenska, Laura Laneque, Hannah Soergel, Claudia Muhle-Goll, Katrin Ochsenreither and Christoph Syldatk
Molecules, 2021, 26 (9), 2759. doi: 10.3390/molecules26092759

Chapter 3 outlines the intensification of the production, *via* lipase-supported biocatalysis, of sorbitol laurate using microwave irradiation in unconventional media. Our "2-in-1" deep eutectic system is therein compared to standardly used organic solvents. This chapter is based on the publication draft:

Intensification of Immobilized Enzyme-Promoted Sugar Alcohol Ester Production in Heterogenous Unconventional Media Using Microwave Irradiation.
André Delavault, Oleksandra Opochenska, Katrin Ochsenreither and Christoph Syldatk
To be submitted

Chapter 4 encompasses the extraction of lipids from oleaginous yeast *Saitozyma podzolica* and the subsequent production of glycolipids using directly the raw oleaginous biomass. Is highlighted the ultra-fast preparation as well, of several DESs using microwave technology. This chapter is based on the publication:

Microwave-Assisted One-Pot Lipid Extraction and Glycolipid Production from Oleaginous Yeast *Saitozyma podzolica* in Sugar Alcohol-Based Media
André Delavault, Katarina Ochs, Olga Gorte, Erwann Durand, Christoph Syldatk and Katrin Ochsenreither
Molecules, 2021, 26 (2), 470, doi: 10.3390/molecules26020470

Chapter 5 concludes on the use of therapeutic Manuka honey to carry the enzymatic glycolipid synthesis as a DES-like medium. The resulting glycolipid-enriched mixtures have been tested against several microorganisms and bioactivity has been characterized using an innovative bio-sensing method.
This chapter is based on the publication draft:

Enhanced Bioactivity of Tailor-Made Glycolipid Enriched Manuka Honey
André Delavault, Ahmed E. Zoheir, Delphine Muller, Kersten S. Rabe, Katrin Ochsenreither, Jens Rudat and Christoph Syldatk
To be submitted

List of Publications

Peer Reviewed Original Publications

Enzymatic Glycolipid Surfactant Synthesis from Renewables
Jens Grüninger*, André Delavault*, Katrin Ochsenreither
Process Biochemistry, 2019, 87, 45–54. doi: 10.1016/j.procbio.2019.09.023
*Co-first authorship

Microwave-Assisted One-Pot Lipid Extraction and Glycolipid Production from Oleaginous Yeast *Saitozyma podzolica* in Sugar Alcohol-Based Media
André Delavault, Katarina Ochs, Olga Gorte, Erwann Durand, Christoph Syldatk and Katrin Ochsenreither
Molecules, 2021, 26 (2), 470, doi: 10.3390/molecules26020470

Lipase-Catalyzed Production of Sorbitol Laurate in a "2-in-1" Deep Eutectic System: Factors Affecting the Synthesis and Scalability
André Delavault, Oleksandra Opochenska, Laura Laneque, Hannah Soergel, Claudia Muhle-Goll, Katrin Ochsenreither and Christoph Syldatk
Molecules, 2021, 26 (9), 2759. doi: 10.3390/molecules26092759

β-Phenylalanine Ester Synthesis from Stable β-Keto Ester Substrate Using Engineered ω-Transaminases
Oliver Buß, Moritz Voss, André Delavault, Pascal Gorenflo, Christoph Syldatk, Uwe Bornscheuer and Jens Rudat
Molecules, 2018, 23 (5), 1211. doi: 10.3390/molecules23051211

Poster Presentations

Enzymatic Conversions of Renewables to Tailor-Made Biotensides in Uncommon Media.
André Delavault, Jens Grüninger, Christoph Syldatk
10th Workshop on Fats and Oils as Renewable Feedstock for the Chemical Industry (2019), Karlsruhe, Germany

Enzymatic Conversions of Renewables to Tailor-Made Glycolipid Surfactants in Uncommon Media.
André Delavault, Jens Grüninger, Christoph Syldatk
1st International Meeting on Deep Eutectic Solvents (2019), Lisbon, Portugal

Enzymatic Conversions of Renewables to Tailor-Made Glycolipids in Uncommon Media.
André Delavault, Jens Grüninger, Christoph Syldatk
Biosurfactants, International Conference (2019), Stuttgart, Germany

Conference Talks

Enzymatically Produced Glycolipids in Deep Eutectic Media
André Delavault, Jens Grüninger, Christoph Syldatk.
2. Meeting: Allianz Biotenside (2018), Aachen, Germany

Problem Solving in Enzymatically Produced Glycolipids
André Delavault, Christoph Syldatk.
3. Meeting: Allianz Biotenside (2019), Ludwigshafen, Germany

Enzymatic Glycolipid Synthesis
André Delavault, Christoph Syldatk.
Final Meeting "SurfGlyco" (2019), Leuna, Germany

Problem Solving in Enzymatically Produced Glycolipids
André Delavault, Christoph Syldatk.
4. Meeting: Allianz Biotenside (2019), Jülich, Germany

Developments Made in Enzymatically Produced Glycolipids
André Delavault, Christoph Syldatk.
5. Final Meeting: Allianz Biotenside (2020), online meeting

Abstract

Glycolipids are promising nonionic surfactants with a wide range of application. They can be encountered daily in cleaning agents, pharmaceuticals or even in food. Due to their versatility and presence in hygiene products, the demand is continuously increasing worldwide. The established chemical synthesis of glycolipids presents several disadvantages such as lack of specificity and selectivity. In contrast to the traditional surfactants from fossil origin, they are not only exhibiting excellent surfactant and emulsifying properties but are also biodegradable and non-toxic to the environment. Glycolipids can be produced by microbial fermentation, by chemical or enzymatical synthesis using renewable resources. In the present work, using lipases, we outline that ester formation can be achieved under mild and nearly water free conditions, the latter being needed to favor condensation instead of the hydrolysis reaction. Thus, using solvents of low water activity, enzymatically synthesized sugar alcohol ester, sorbitol laurate, was used as a model product. Consequently, this dissertation highlights enzymatic synthesis as a method of choice for the tailor-made production of a potential wide range of novel surfactants. Biocatalysis enabled an innovative approach, in respect to current concerns on sustainability, of this topical subject with a multidisciplinary approach.

The solubility of polyols such as sugars or sugar alcohols in organic solvents is rather low. The enzymatic synthesis of these compounds is however possible in nearly water free media using inexpensive and renewable building blocks. We propose initially a "2-in-1" system that overcomes solubility problems, as a Deep Eutectic System (DES) made of sorbitol and choline chloride replaces an either purely organic or aqueous medium. For the first time, 16 commercially available lipase formulations were compared and factors affecting the conversion were investigated to optimize this process owing to a newly developed HPLC-ELSD method for quantification. Thus, using 50 g/L of lipase formulation Novozym 435® at 50°C, the optimized synthesis of sorbitol laurate (SL) allowed to achieve 28% molar conversion of 0.5 M of vinyl laurate to its sugar alcohol monoester when the DES contained 5 wt.% water. After 48h, the *de novo* synthesized glycolipid was separated from the media by liquid-liquid extraction, purified by flash-chromatography and characterized by one- and two-dimensional Nuclear Magnetic Resonance (NMR) experiments and Mass Spectrometry (MS). In completion, we provide an initial proof of scalability for this process. Using a 2.5 L stirred tank reactor (STR) allowed a batch production reaching 25 g/L in a highly viscous two-phase system.

Many challenges remain still, and limit expectations of industrial application. Extensive time of production and relatively low initial reaction velocities are among the barriers that need to be raised. We focused, in this context, on the intensification of the reaction velocities and yields, for the production of sorbitol laurate owing to a microwave-assisted method that we, nonetheless, compared to a conventional heating one. Different solvent systems were also put in competition, in which 2M2B revealed itself as a more performant solvent system than the DES. Thus, using 20 g/L of immobilized lipase formulation, 0.75 M of vinyl laurate, 0.25 M of sorbitol at 50°C in 2M2B allowed to reach 92% of conversion yield which corresponds to a 168 g/L titer production. Use of microwave technology allowed thereafter a 50-fold increase of the initial reaction rate in the organic solvent at 75°C. Re-use of the biocatalyst was studied as well in 2M2B and DES, highlighting in the latter a greater destabilization of the enzyme. We bring thus, nuance to the intense build-up DESs are currently undergoing, in literature, as media for

biocatalysis. Nevertheless, we indicate encouraging prospects of betterment to make DESs competitive to standard media for bioconversions. In completion, we investigated the efficiency of our downstream processing that allows complete recovery of sorbitol laurate after 4 cycles of liquid-liquid extraction.

Existing reports describing integrated processes for glycolipid production from renewables use many reaction steps, therefore we aimed at simplifying the procedure. By using microwave dielectric heating, DESs preparation was first accelerated considerably. Our comparative study revealed a preparation time on average 16-fold faster than the conventional heating method in an incubator. Furthermore, lipids from robust oleaginous yeast biomass were successfully extracted up to 70% without using the pretreatment method for cell disruption, limiting logically the energy input necessary for such process. Acidified DESs consisting of either xylitol or sorbitol and choline chloride mediated the one-pot process, allowing subsequent conversion of the lipids into mono-acylated palmitate, oleate, linoleate, and stearate sugar alcohol esters. Thus, we show strong evidence that addition of immobilized *Candida antarctica* Lipase B (Novozym 435®), in acidified DES mixture, enables a simplified and fast glycolipid synthesis using directly oleaginous yeast biomass.

Finally, we demonstrate that the DES-like Manuka honey can also serve as a medium for glycolipid synthesis. Indeed, this supersaturated sugar solution can be compared in terms of physicochemical properties to the sugar based DESs. Honey products being commercially available for therapeutic applications, it appears interesting to enhance its bioactivity. In the final chapter of this dissertation, we investigate if enriching medical grade honey with *in situ* enzymatically synthetized glycolipids can improve the antimicrobial property of the mixture. Tested mixtures are composed of Manuka honey enriched with octanoate, decanoate, laurate and myristate sugar esters respectively dubbed GOH, GDH, GLH and GMH. To characterize the bioactivity of those mixtures, first a qualitative screening using agar well diffusion assay has been performed with methicillin-resistant *Staphylococcus aureus*, *Bacillus subtilis*, *Candida bombicola*, *Escherichia coli* and *Pseudomonas putida* which confirmed a considerably enhanced susceptibility of these micro-organisms to the different glycolipid enriched honey mixtures. A designed biosensor *E. coli* strain that displays a stress reporter system consisting of three stress-specifically inducible, red, green and blue fluorescent proteins which respectively translate physiological stress, genotoxicity and cytotoxicity was then used. The latter allowing bioactivity characterization and showed a six-fold enhancement of the physiological stress caused by GOH compared to regular Manuka honey at 1.6% (v/v) concentration. Antibacterial agar well diffusion assay with *E. coli* was then performed and demonstrated an improved inhibitory potential with GOH upon 20% (v/v) concentration.

Zusammenfassung

Glykolipide sind vielversprechende nichtionische Tenside mit einem breiten Anwendungsspektrum. Man begegnet ihnen täglich in Reinigungsmitteln, Pharmazeutika oder auch in Lebensmitteln. Aufgrund ihrer Vielseitigkeit und Präsenz in Hygieneprodukten steigt die Nachfrage weltweit kontinuierlich an. Die etablierte chemische Synthese von Glykolipiden weist einige Nachteile wie mangelnde Spezifität und Selektivität auf. Im Gegensatz zu den traditionellen Tensiden fossilen Ursprungs weisen sie nicht nur hervorragende Tensid- und Emulgiereigenschaften auf, sondern sind auch biologisch abbaubar und nicht toxisch für die Umwelt. Glykolipide können durch mikrobielle Fermentation, durch chemische oder enzymatische Synthese unter Verwendung erneuerbarer Ressourcen hergestellt werden. In der vorliegenden Arbeit zeigen wir unter Verwendung von Lipasen, dass die Esterbildung unter milden und nahezu wasserfreien Bedingungen erreicht werden kann, wobei letztere erforderlich sind, um die Kondensation anstelle der Hydrolysereaktion zu begünstigen. So wurde unter Verwendung von Lösungsmitteln mit geringer Wasseraktivität der enzymatisch synthetisierte Zuckeralkoholester, Sorbitollaurat, als Modellprodukt verwendet. Folglich hebt diese Dissertation die enzymatische Synthese als Methode der Wahl für die maßgeschneiderte Herstellung einer potenziell breiten Palette von neuartigen Tensiden hervor. Die Biokatalyse ermöglichte eine innovative Herangehensweise an dieses aktuelle Thema mit einem multidisziplinären Ansatz im Hinblick auf die aktuellen Bedenken zur Nachhaltigkeit.

Die Löslichkeit von Polyolen wie Zuckern oder Zuckeralkoholen in organischen Lösungsmitteln ist eher gering. Die enzymatische Synthese dieser Verbindungen ist jedoch in nahezu wasserfreien Medien unter Verwendung kostengünstiger und nachwachsender Rohstoffe möglich. Mit Hilfe von Lipasen kann die Esterbildung unter milden Bedingungen erreicht werden. Wir schlagen zunächst ein "2-in-1"-System vor, das die Löslichkeitsprobleme überwindet, da ein Deep Eutectic System (DES) aus Sorbitol und Cholinchlorid entweder ein rein organisches oder wässriges Medium ersetzt. Erstmals wurden 16 kommerziell erhältliche Lipase-Formulierungen verglichen und Faktoren, die den Umsatz beeinflussen, untersucht, um diesen Prozess dank einer neu entwickelten HPLC-ELSD-Methode zur Quantifizierung zu optimieren. So konnte bei der optimierten Synthese von Sorbitollaurat (SL) unter Verwendung von 50 g/L der Lipaseformulierung Novozym 435® bei 50°C eine 28%ige molare Umsetzung von 0,5 M Vinyllaurat zu seinem Zuckeralkoholmonoester erreicht werden, wenn das DES 5 Gew.-% Wasser enthielt. Nach 48h wurde das *de novo* synthetisierte Glykolipid durch Flüssig-Flüssig-Extraktion vom Medium abgetrennt, durch Flash-Chromatographie gereinigt und durch ein- und zweidimensionale Kernspinresonanz (NMR)-Experimente und Massenspektrometrie (MS) charakterisiert. Abschließend erbringen wir einen ersten Nachweis der Skalierbarkeit für diesen Prozess. Die Verwendung eines 2,5-L-Rührkesselreaktors (STR) ermöglichte eine Batch-Produktion bis zu 25 g/L in einem hochviskosen Zweiphasensystem.

Dennoch bleiben viele Herausforderungen bestehen und begrenzen die Erwartungen an eine industrielle Anwendung. Eine lange Produktionszeit und relativ niedrige Anfangsreaktionsgeschwindigkeiten gehören zu den Barrieren, die es zu überwinden gilt. Wir konzentrierten uns in diesem Zusammenhang auf die Steigerung der Reaktionsgeschwindigkeiten und Ausbeuten bei der Herstellung von Sorbitollaurat durch eine mikrowellenunterstützte Methode, die wir jedoch mit einer konventionellen

Heizmethode verglichen. Es wurden auch verschiedene Lösungsmittelsysteme miteinander konkurriert, wobei sich 2M2B als ein leistungsfähigeres Lösungsmittelsystem als das DES erwies. So erlaubte die Verwendung von 20 g/L immobilisierter Lipaseformulierung, 0,75 M Vinyllaurat, 0,25 M Sorbitol bei 50°C in 2M2B eine 92%ige Konversionsausbeute zu erreichen, was einer 168 g/L Titerproduktion entspricht. Der Einsatz der Mikrowellentechnologie ermöglichte danach eine 50-fache Steigerung der ursprünglichen Reaktionsgeschwindigkeit im organischen Lösungsmittel bei 75°C. Die Wiederverwendung des Biokatalysators wurde auch in 2M2B und DES untersucht, wobei bei letzterem eine größere Destabilisierung des Enzyms festgestellt wurde. Wir bringen somit eine Nuance in die intensive Entwicklung, die DESs derzeit in der Literatur als Medium für die Biokatalyse erfahren. Nichtsdestotrotz zeigen wir ermutigende Aussichten auf Verbesserungen, um DESs konkurrenzfähig zu Standardmedien für Biokonversionen zu machen. Abschließend untersuchten wir die Effizienz unseres Downstream-Prozesses, der die vollständige Rückgewinnung von Sorbitollaurat nach 4 Zyklen der Flüssig-Flüssig-Extraktion ermöglicht.

Bestehende Berichte, die integrierte Prozesse zur Glykolipidproduktion aus nachwachsenden Rohstoffen beschreiben, verwenden viele Reaktionsschritte. Daher zielten wir darauf ab, das Verfahren zu vereinfachen. Durch die Verwendung von dielektrischer Mikrowellenerwärmung wurde die Herstellung von DESs zunächst erheblich beschleunigt. Unsere Vergleichsstudie ergab eine durchschnittlich 16-fach schnellere Vorbereitungszeit als die konventionelle Erhitzungsmethode in einem Inkubator. Darüber hinaus konnten Lipide aus robuster ölhaltiger Hefe-Biomasse erfolgreich bis zu 70% extrahiert werden, ohne die Vorbehandlungsmethode für den Zellaufschluss zu verwenden, wodurch der für einen solchen Prozess notwendigen Energieaufwand begrenzt. Angesäuerte DESs, die entweder aus Xylit oder Sorbit und Cholinchlorid bestanden, vermittelten den Eintopfprozess und ermöglichten die anschließende Umwandlung der Lipide in monoacylierte Palmitat-, Oleat-, Linoleat- und Stearat-Zuckeralkoholester. Somit zeigen wir, dass die Zugabe von immobilisierter *Candida antarctica* Lipase B (Novozym 435®) in einer angesäuerten DES-Mischung eine vereinfachte und schnelle Glykolipidsynthese unter Verwendung direkt ölhaltiger Hefebiomasse ermöglicht.

Schließlich stellen wir fest, dass der DES-ähnliche Manuka-Honig auch als Medium für die Glykolipidsynthese dienen kann. Diese übersättigte Zuckerlösung kann in Bezug auf die physikochemischen Eigenschaften mit den zuckerbasierten DESs verglichen werden. Da Honigprodukte für therapeutische Zwecke kommerziell verfügbar sind, erscheint es interessant, ihre Bioaktivität zu erhöhen. Im letzten Kapitel dieser Dissertation wird untersucht, ob die Anreicherung von medizinischem Honig mit in situ enzymatisch synthetisierten Glykolipiden die antimikrobielle Eigenschaft der Mischung verbessern kann. Die getesteten Mischungen bestehen aus Manuka-Honig, der mit Octanoat-, Decanoat-, Laurat- und Myristat-Zuckerestern angereichert ist, die jeweils als GOH, GDH, GLH und GMH bezeichnet werden. Um die Bioaktivität dieser Mischungen zu charakterisieren, wurde zunächst ein qualitatives Screening mittels Agar-Well-Diffusionstest mit Methicillin-resistentem *Staphylococcus aureus*, *Bacillus subtilis*, *Candida bombicola*, *Escherichia coli* und *Pseudomonas putida* durchgeführt, das eine deutlich erhöhte Empfindlichkeit dieser Mikroorganismen gegenüber den verschiedenen mit Glykolipiden angereicherten Honigmischungen bestätigte. Dann wurde ein entworfener Biosensor *E. coli*-Stamm verwendet, der ein Stressreportersystem aufweist, das aus drei stressspezifisch induzierbaren, rot, grün und blau fluoreszierenden Proteinen besteht, die jeweils physiologischen Stress,

Genotoxizität und Zytotoxizität übersetzen. Letzteres ermöglichte die Charakterisierung der Bioaktivität und zeigte eine sechsfache Steigerung des physiologischen Stresses durch GOH im Vergleich zu regulärem Manuka-Honig bei einer Konzentration von 1,6% (v/v). Der antibakterielle Agar-Well-Diffusionstest mit *E. coli* wurde dann durchgeführt und zeigte ein verbessertes hemmendes Potential mit GOH bei 20% (v/v) Konzentration.

Table of Content

1. Theoretical Background and Research Proposal

This chapter is partly based on the publication:

Enzymatic Glycolipid Surfactant Synthesis from Renewables
Jens Grüninger*, André Delavault*, Katrin Ochsenreither
Process Biochemistry, 2019, 87, 45–54
doi: 10.1016/j.procbio.2019.09.023
Published: 20.09.2019
*Co-first authorship

Authors contributions:

Jens Grüninger and André Delavault: Conceptualization, writing original draft, review and editing of the final manuscript

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

SURFace ACTive AgeNTS or SURFACTANTS are amphiphilic molecules able to form micelles and thereby reduce the surface tension of aqueous media (e.g., air-water), and the interfacial tension of liquid-liquid (e.g., oil-water or water-oil) or liquid–solid (e.g., wetting phenomena) systems. Efficient surfactants can lower the surface tension of water from 72 to 30 mN/m and interfacial tension for water against *n*-hexadecane from 40 to 1 mN/m [1].

1.1. Surfactants

Surfactants can be found in many products of daily life like household detergents and soaps [1] and are widely used in industry for agrochemical, food, cosmetic and pharmaceutical applications assisting the production of foam, emulsions and dispersions [2,3]. Surfactants are generally composed of a polar hydrophilic head group and a nonpolar hydrophobic tail. Due to the nature of both groups, they can be classified according to Hoffmann & Ulbricht [4] as non-ionic, anionic, cationic and amphoteric surfactants. Due to synergistic effects, amphoteric surfactants are often used together with anionic surfactants in hair shampoos and other cosmetic products, because the skin compatibility of anionic surfactants is significantly improved by the addition of amphoteric surfactants. However, most of these compounds are of synthetic origin, chemically produced from crude oil, are not fully biodegradable and potentially exhibit environmental problems due to their persistent nature [5]. Most of the surfactants are removed in sewage treatment plants, but some of them unfortunately still end up in surface waters, soils, or sediments. Concern about the accumulation of surfactants in the environment is growing. Acute toxicity has already been reported against some bacterial species, aquatic plants, invertebrates and vertebrates [6].

Therefore, the trend today is going towards the use of eco-friendly or "green" surfactants originating from renewable resources (see Chapter 4). A surfactant can be described as a biosurfactant if it originates from microorganisms and was obtained by a separation process (extraction, precipitation, or distillation). Glycolipids are the best known group within the biosurfactants [7]. In principle, it is also possible to manufacture a biobased surfactant by combining chemical and biotechnological methods consisting of a naturally derived hydrophobic part, e.g. a long-chain fatty acid, hydroxyl fatty acid or an alpha-alky-β-hydroxy fatty acid, and a hydrophilic part, e.g. a carbohydrate, an amino acid, a cyclic peptide, a phosphate, a carboxylic acid or alcohol, etc. [8]. As all glycolipids are of non-ionic nature, the classification of the chemically produced surfactants is not able to distinguish between the different glycolipids of biotechnological processes. Therefore, they are usually named after their polar head group consisting in this case of a varying saccharide.

The development of ecological products and solvents is one of the most important research areas of Green Chemistry. This is a vast challenge with multidisciplinary issues because the regular use of organic solvents represents most of the waste in syntheses and industrial processes. In this context, it is essential to find alternatives to non-ecological solvents that will carry the synthesis of biodegradable products based on renewables. Such problematic refers directly to several principles of green chemistry and nowadays concerns. To this end, non-ionic surfactants such as glycolipids are a good example of bio-based and non-persistent products.

1.2. Microbial Glycolipids – an Overview

Fatty acid sugar esters also known as glycolipids are a class of surfactants, based on a sugar moiety and a fatty acid tail. Synthesis of glycolipids can be performed by chemical processes as well as by microbial or enzymatic conversions [9]. Microbial glycolipids consist of one or more saccharides as hydrophilic part and different fatty acids as hydrophobic tail. The fatty acids can be ramified or unramified, the chain length is variable and can contain different functional groups. This variability leads to a large range of different glycolipids. Microbial glycolipids have very good surfactant and emulsifier characteristics, are biodegradable and non-toxic to the environment [9]. Being odor- and tasteless and non-irritant, they are discussed to be very interesting for applications as emulsifier in food or cosmetic formulations [10,11]. Glycolipids are effective against microbes [12], viruses [13] and fungi [14]. They are also considered as potential anti-tumour agents [15], so that they may be interesting for the pharmaceutical industry. Rhamnolipids produced by *Pseudomonas aeruginosa* are among the best-known microbial glycolipids. Furthermore, sophorolipids (SL) produced by *Candida bombicola*, mannosylerythritollipids (MEL) by *Pseudozyma* species and cellobioselipids (CL) by *Cryptococcus humicola* and *Pseudozyma fusiformata* [3,16] are well described glycolipids in literature.

Microbial fermentation processes have been commercialized for the production of RLs, SLs and MELs leading to highly active and sometimes even bioactive glycolipids exhibiting very low CMC values. The company Evonik produces SLs in a production plant in Slovakia and starts the production of RLs, it is also planned to produce the latter *via* bacterial fermentation instead of yeasts. The natural diversity of biosurfactants, can be further enhanced by chemical or enzymatic post-treatment to obtain glycolipid surfactants with modified properties. By using different enzymes novel structures with different properties can be obtained, which might have the potential to alter and improve the physico-chemical characteristics of known glycolipids to better fit any desired application [17,18]. As reported previously, different degrees and positions of acetylation within MELs play a key role to some physico-chemical properties of these glycolipids [19], thus being an interesting target for further modification.

The *de novo* synthesis of glycolipids by microbial fermentation has the advantage that a whole range of different, renewable substrates or also side- and waste streams from other industries can be used as substrates which do not have to be components of the end product. Microbial glycolipids can thereby contain rare or unusual fatty acids or sugars which cannot be obtained chemically. By using side- or waste-streams overall process economy can be improved, and production of wastes can be reduced. Microbially produced glycolipids are often mixtures of different alkyl-chain length or of different branched alkyl chains. As medium composition can also influence the product spectrum, using non-defined waste substrates of varying composition, can result in different product mixtures. To obtain a standardized product, a complex purification step (e.g., by chromatography) must be integrated in the downstream process. This results in the isolation of a product with comparably well-defined properties but also increases the price of product due to expensive purification.

1.3. (Bio-)Chemical Production of Glycolipids from Renewables

Monosaccharides and polysaccharides, which are obtained from renewable resources, belong to a substance class which gains more and more importance as source material for surfactants. Surfactants based on sugars are long known and were used as emulsifiers in special technical

laundry detergents. The large-scale production of sugar tensides in the field of laundry- and cleaning supplies as co-tensides, however, is still a relatively new development. Ethers of glucose (or other sugars) with a residue (the aglycon) bound to the sugar are described as glucosides or alkylpolyglucosides (APGs). These APGs find diverse applications in personal care products, cosmetics, extraction of organic dyes as well as in membrane protein research. APGs belong to nonionic tensides and are better surfactants than Triton X-100 because of their excellent behavior at interfaces [20][21], their chemical stability in water and especially under alkaline conditions and their non-reactivity towards oxygen [22]. The glucoside consists of a specific sugar which acts as a hydrophilic head consisting mainly of a mono- or disaccharide, e.g., glucose, galactose, maltose, xylose or rhamnose. The properties of the glucoside depend on the structure and bond between the oligomeric head groups [23]. The hydrocarbon tail consists of a primary alcohol of different chain length. It can be either saturated or unsaturated [22,24–27]. APGs are established surfactants industrially produced by Fischer glycosylation (direct synthesis or transacetylation) on a large scale, offering good yields at a low price because the starting materials are cheap to purchase on a large scale. However, chemical synthesis leads to the formation of a product mixture with very different properties [28]. Further disadvantages of this process are the need of high temperatures, toxic catalysts and multiple steps of protection, deprotection and activation [29] (for further details see subsection 1.9.).

Although renewable raw materials cover currently only about ten percent of the chemical industry's raw material requirements, oleochemicals account for a large proportion. Therefore, most fatty acids are already derived from plant oils since decades. One example is palm oil, which is rich in palmitic and oleic acid. Other examples are linseed and castor oil, which are almost exclusively used for industrial processes. Lauric acid obtained from palm kernel and coconut oil is the most important feedstock to produce surfactants. In addition to lauric and myristic acid palm kernel and coconut oil additionally contains capric and caprylic acid which are also used in the chemical industry as alkyl chains for the production of surfactants [30].

In contrast to multi-stage chemical processes in which catalysts often must be added, regioselective synthesis takes place under very gentle reaction conditions. The direct coupling between sugar and fatty alcohol enables the production of previously unknown glycolipids. Therefore, enzymes with hydrolytic properties are used under non-aqueous conditions in which their hydrolytic activity is reversed to condensation. In addition, high substrate concentrations are advantageous to shift the reaction equilibrium to the product side. Thus, glycosidic bonds can be formed. Proof-of-concept studies have been successfully conducted for enzymes of the lipase and β-glucosidase classes. These enzymes are discussed in more detail in subsections 1.7. and 1.8. The theoretical combination of nearly all sugars with all fatty acid derivatives enables a very large variety of glycolipid surfactants to be formed enzymatically. Therefore, it is important to understand and investigate enzymatic synthesis processes, which should ultimately enable the production of tailor-made glycolipids. Nevertheless, a biotechnological production on a commercial scale has only been realized in very few cases, and up to now products are only found in niche applications due to their distinctly higher prices compared to traditional surfactants. Regardless of glycolipids being produced chemically or enzymatically, it is important to understand in which way hydrophilic and hydrophobic parts influence their properties to enable a rational design of surfactants.

1.4. Structure-Activity Relationship of Glycolipids

Structure-activity relationship is a key element toward understanding the link between a chemical structure and its activity, whether it is biological or physical. In the case of glycolipids the main physico-chemical parameters to measure function/application are the Critical Micelle Concentration (CMC), surface tension and interfacial tension which are often referenced as "surfactant activity", which represent the triumvirate of glycolipid characterization [31]. Hydrophilic-Hydrophobic Balance is also often determined but since its calculation is based on the molar mass it can only serve as an orientation for glycolipid properties [32].

Structure-activity relationship of a glycolipid is in principle determined by the interplay of hydrophilic head group, hydrophobic tail and the type of linkage between both parts. Regarding the hydrophilic head group, major determinants are type and number of saccharides influencing the size of the hydrophilic part. In case of the hydrophobic tail, the lengths of the carbon chain, the number of tails and the ramifications on this / these tail/s are the adjustments of the apolar part that can modify the overall properties. The linker in a glycolipid can be an ester, ether, thioester, amine or amide function [33]. It seems evident that the type of linkage is crucial for the stability and indirectly the biodegradability of the biosurfactant, so that the stability of the chemical function dictates the degradability level of the surfactant [34]. Although the number of combinations seems endless, only three effects appear to be major: the alkyl chain length, the number of tails as well as the type of linkage.
According to the literature, the CMC value of a glycolipid in an aqueous solution is mainly influenced by the length of the alkyl chain and declines as the length of the chain increases [35,36]. As described by Ferrer *et al.* as a rule of thumb, CMC values of a glycolipid decrease by about 1 unit when carbon chain length increases by 6 atoms, regardless of the sugar moiety present on the structure [37]. Due to its relatively small size compared to the hydrophobic part, the type of saccharide seems not to exert an significant influence of surface activity [37]. Concerning the number of acylation, experiments showed that a second tail branched to the sugar tends to decrease the surface activity compared to monoesters. Conjecture is here, that a higher degree of acylation opens the surfactant structure and therefore leads to less efficient aggregation at the surface [38,39]. Finally, the type of linkage determines the space between the hydrophobic and hydrophilic units. As the alkyl chain gets closer to the carbohydrate, the hydrophobic effect of the tail gets inhibited resulting into a loss of hydrophobicity [40]. A shorter results therefore in a higher CMC value [34].

1.5. Solvent Systems and Reaction Conditions

Solvents are, by definition, substances in which gases, liquids or solids are dissolved without taking part in the chemical reaction. Enzyme reactions usually take place in water as a solvent in which, in addition to the enzymes themselves, salts and organic compounds such as sugars, organic acids, amino acids, and lipids are homogeneously dissolved. Water is in many enzyme reactions not only solvent, but it is also reactant and substrate, e.g., in hydrolysis.

In the 1980s, it was proven that many enzymes of the enzyme class EC 3 (hydrolases) which were present in almost water-free, nonpolar solvents catalyze the reverse hydrolysis reaction, i.e. condensation instead of the hydrolysis [41,42]. The condensation reaction can be used for enzymatic glycolipid synthesis. Although the solvent should have low water activity to prevent hydrolysis, the water layer bound to the enzyme is essential to keep the enzyme in an active state, therefore, it should not be affected by the solvent [43,44]. An enzymatic synthesis

is only possible as soon as a suitable combination of enzyme and solvent and corresponding water activity is found. Therefore, several experiments have been performed to reduce the water content and the water activity of the reaction mixture before and during the reaction. In the study of Chamouleau *et al.* molecular sieves (4A) were used to remove the water from the reaction medium resulting in a constantly low water activity during the entire reaction. It is also possible to adjust the desired water activity of the solvent by pre-incubation with saturated salt solutions [45]. To avoid the formation of water during the reaction, a transesterification could be pursued instead of a reversed hydrolysis, using fatty acid esters instead of free fatty acids. The molar ratio between sugar and fatty acid is another parameter influencing the enzymatic synthesis of glycolipids. Using fatty acids or fatty alcohols in excess might influence the reaction rate and the yield of the experiment, since free fatty acids or alcohols can have an inhibiting effect on the enzyme [45]. Furthermore, solubility of the reactants in the solvent system must be considered. Regardless of the solvent system, elevated temperatures improve solubility of reactants, but might impair stability and selectivity of the enzyme possibly preventing glycolipid production. Thermostable, immobilized lipases are already available from many suppliers covering a wide temperature range between 20° and 60°C being stable over the entire process. However, depending on the solvent, elevated temperature might be problematic.

Organic solvents have been frequently used for enzymatic glycolipid synthesis reactions as they are almost water-free and of low water-activity favoring the condensation reaction. However, organic solvents display high volatility, which is troublesome for an eco-friendly process Furthermore, the sugar moiety of a glycolipid surfactant will be highly soluble in water but a lot less in an organic solvent and the opposite will be observed for the fatty acid tail. This allows a very large scope to find the optimal conditions for the different glycolipid syntheses [46,47]. In order to tackle these problematics, ionic liquids (ILs) were used to overcome the solubility problems inherent to both substrates in either hydrophobic or hydrophilic media [48]. It has been widely accepted that certain ILs, especially the ones containing imidazolium anions, enhance the enzymes´ activity [49]. However, nowadays Deep Eutectic Solvents (DES) are often preferred over ILs for reasons that will be discussed further in the coming section.

Among other alternative solvents, liquid CO_2 is promising. Indeed, lipase catalyzed acylation can be greatly optimized in this supercritical fluid [50]. Even more original and innovative, the combination of ILs and supercritical CO_2 results in a mixed biphasic system that can be used for lipase catalyzed synthesis of sugar esters and reaches conversion rates higher than both technologies taken separately (up to 95.5%) [51]. Biphasic systems can also be adapted to DES but this kind of combination has been only successfully applied for protein extraction yet [52]. Compared to ILs and supercritical CO_2, eutectics have not been proven yet to be the most efficient solvent to carry lipase catalyzed transesterification [53]. However, there is still room for improvement and innovation in the DES domain. Many of the problems encountered with DES that are related to physico-chemical barriers can be overcome as the technologies that comes with it (e.g., stirring systems, heating systems…) are also evolving. Therefore, an innovative technology such as DES should also be used with innovative reactors and energy sources.

1.6. Deep Eutectic Solvents (DES): a Cheap, Biodegradable, and Uncommon Media Made from Renewables

First described by Abbott *et al*. in 2002, DESs are a quite new solvent system compared to their older relatives, ILs [54]. They consist of an ammonium or phosphonium salt and one or a mixture of hydrogen bond donors (HBD) like alcohols, amines, amides or a carboxylic acid [55]. When these components, also called DES partners, are mixed and heated in the right molar ratio, they develop the ability to form a stable liquid phase at room temperature. Inside the DES on a molecular level the Hydrogen Bond Donors (HBDs) are forming hydrogen bonds between them but also with the other cationic and anionic species thus creating a tridimensional network. As a result on the macromolecular level, drastic decreases of the melting points of the components are observed and a liquid phase stable at room temperature is obtained [56,57] (**Figure 1**).

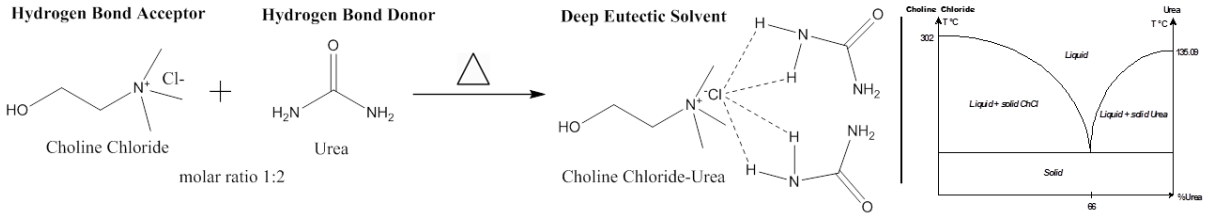


Figure 1. Formation of a Deep Eutectic Solvent (DES) and phase diagram.

In the continuity of these solvents, another term emerged: Natural Deep Eutectic Solvent (NaDES). More recent, this NaDES term describes a liquid obtained by combining molecules abundantly present in nature, even though the line between NaDES and DES is still not clearly drawn [58]. However, either called DES or NaDES, the principle of eutectic media is a promising alternative to traditional solvents and ILs. Moreover, in addition to being over-represented in the literature their non-toxicity is very often questioned. Indeed, some ILs seem to represent a real threat to soils and aquatic environments [59].

In contrast, DESs are generally composed of two or three safe and inexpensive components, which are involved in hydrogen bond interactions with each other to obtain a eutectic mixture with a melting point much lower than that of each component species [60]. Since, components of a standard DES include for example choline chloride (ChCl), urea, glycerol, natural carboxylic acids, amino acids, carbohydrates, polyalcohols and related carbonated structures that are ubiquitous in nature, DESs display low toxicity, high thermal stability, high biodegradability, high recyclability, low inflammability and volatility [54,61]. Their physico-chemical properties can be modulated by different combinations of molecules and as a result they can serve diverse and varied applications making them a versatile and interesting media for biochemistry.

1.7. Lipases in Glycolipids Production

Lipases (water soluble triacylglycerol acylhydrolases, EC 3.1.1.3) have been commonly used for acylation reactions over decades. Their enantioselective and regioselective nature make them an important tool that is often applied in modern organic synthesis, for example in enantiomeric resolutions [62]. These versatile biocatalysts used in different industrial domains are obtained from plants, animals and wild-type or recombinant microorganisms. They do not require a cofactor for their catalytic activity and benefit a broad range of substrate spectrum.

Enzymatic reactions using lipases have been tuned toward industry to produce a variety of different compounds such as fine medicinal chemicals, polymeric materials and biofuels. Because of their high stability and activity, lipases are since the 1980´s, a popular tool in the industry [63].

Lipases have been used extensively for over 30 years for enzymatic synthesis of glycolipids (**Figure 2**) due to their advantageous properties and versatility. According to literature, the first experiment using lipase was reported by Seino *et al*. [64], followed by Chopineau *et al.* [65] and Therisod and Klibanov [66]. The application of a brand-new method was motivated to overcome coloring of products and solvent toxicity problems in the chemical process for the industrial sucrose acylation. It was also expected that mono-, di- or triesters could be obtained selectively if the substrate specificity of the enzyme would be affected on the primary groups of the sucrose. Such initiative resolved the problems of occurrence of toxic side products and products coloring but also induce the regioselective acylation of the sugars [64].

Until nowadays, many experiments have been carried out with different conditions such as other lipases, solvents, sugars, and fatty acids. Despite an extensive utilization of the enzymatic reaction, a universal reaction system and lipase has not been established for the synthesis of glycolipid surfactants yet.

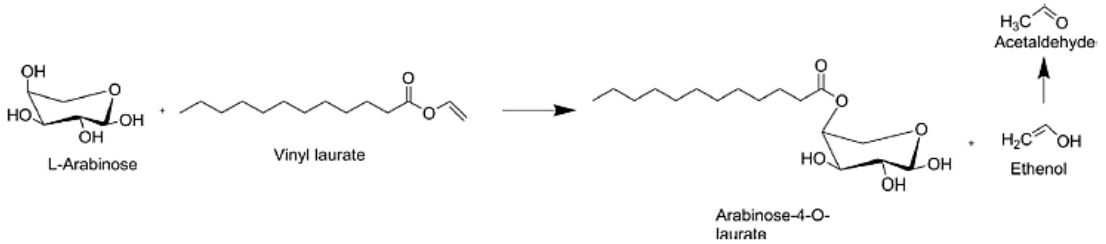


Figure 2. Example of lipase catalyzed reaction for glycolipid production

Among the first enzymes that have been used toward glycolipid synthesis, the PPL (Pancreatic Porcine Lipase) can be encountered several times [65,66]. Showing good activity and stability even at high temperatures, it is also one of the cheapest commercially available non-microbial lipase [67]. Nevertheless, the PPL presents the serious disadvantage to often contain impurities like esterases, trypsin and other proteases which result in a loss of specificity and occurrence of side products [68]. Microbial lipases like those from *Rhizomucor miehei*, *Candida rugosa*, *C. antartica*, *Burkholderia sp.* or *Pseudomonas sp.* are nowadays mainly used. In parallel to this, immobilization of lipases has been an optimized strategy to improve the stability and facilitate the purification. In the meantime, this strategy also enables the re-utilization of the biocatalyst [69]. The process of immobilization generally reduces the activity of the enzyme, because of active site obstruction, introduction of a tremendous amount of non-catalytic ballast in the reaction, albeit the activity of immobilized CAL-B remains high [70].

Production of fructose and glucose esters of stearic acid was conducted by the use of lipases from *R. miehei* in tertiary butyl alcohol with yields between 10% and 24% [71]. Also, Khaled *et al.* managed to reach yields up to 83% using a fixed-bed reactor for the synthesis of fructose oleate in 2-methyl-2-butanol (2m2b) [72]. In 2006 it has been shown that compared to two immobilized *C. antarctica* lipases, the immobilized Lipozyme IM from *R. miehei* had a much lower conversion rate [73]. Another reported enzyme also often used is the lipase from *C. rugosa*. After immobilization, this lipase reached over 70% conversion rate when applied to the synthesis of lactone caprate in acetone after a 48h reaction time [74].

However, one of the most used lipases in organic synthesis processes is the lipase from *C. antarctica* type B (CAL-B). This enzyme is commercially available in various immobilized forms but also free from petroleum-derived carrier. The immobilized version, iCAL-B, is composed of an acrylic resin and sold under the name "Novozym 435", but several other materials for immobilization like silica, chitin, or agarose were used. Pöhnlein *et al.* [75] compared seven different lipases in six organic solvents. Novozym 435 was the most efficient lipase and showed transesterification activity in five of the tested organic solvents. Furthermore, in four of these, it was possible to synthesize glucose octanoate. **Table 1** shows different yields for a wide range of sugar ester syntheses using mostly Novozym 435 in a variety of media providing a good general overview of the different structures of glycolipids that can be obtained by lipase-catalyzed reaction.

Table 1. Overview of lipase-catalyzed synthesis of glycolipid surfactants in various solvents.

Sugar	Fatty acid	Lipase	Solvent(s)	Yield (%)	References
Fructose	Palmitic acid	Novozyme 435	2m2b and THF	66	[76]
Fructose	Lauric acid	Novozyme 435	Ethyl-methylketone	82	[73]
Fructose	Palmitic acid	Novozyme 435	SC CO_2	Up to 74	[77]
Glucose/xylose	Vinyl octanoate	Novozyme 435	DES	4.8	[78,79]
Glucose	Vinyl laurate	Novozyme 435	ILs	Up to 86	[80]
Xylose	Caprylic acid	Novozyme 435	DMSO:acetone (1:10)	64	[81]
Glucose	Myristic acid	CAL-B	2m2b	43	[82]

Abbreviations: 2m2b, methyl-2-butanol; THF, tetrahydrofuran; SC CO_2, supercritical carbon dioxide; DES, deep eutectic solvent; IL, ionic liquid.

Apparently, yields superior to 80% can be achieved in greener organic solvents (e.g. butanone) [83] and also in ILs [80] but syntheses in DES still result in poor yields not suitable for industrial application. Despite showing promising developments for a renewable synthesis and low production costs, biocatalysis in deep eutectic systems will need a lot more research efforts to be carried out to the industrial scale. However, since DES systems can be already be found in industry (mostly for metal finishing) [84] and since it is also possible to produce them on the kilo scale with continuous flow with mechanochemistry techniques [85], industrial glycolipid synthesis in DES seems to be feasible. Such techniques and technologies, like microwave reactors as well, might be a key to help biocatalysis in deep eutectic systems to grow in the industrial world. Although the ways of improvement and the challenges are numerous, scaling-up lipase-catalyzed synthesis for glycolipids production gives dynamism to biocatalysis since these target compounds represent a huge turnover for the chemical industry. This aspect will be further discussed in part 4.

More recently, Siebenhaller *et al.* found a new and sustainable reaction system for the lipase catalyzed synthesis of glycolipids (**Figure 2**). This process minimizes the usage of hazardous and environmental harmful organic solvents. This innovative system uses a DES media based

on choline chloride and a sugar; the latter can also be replaced with a sugar alcohol, which acts as the hydrogen bond donor [78]. Five sugars, three sugar alcohols and one anhydrosugar in combination with up to nine different fatty acids were tested, showing the versatility of such a process. By this means, the tailor-made enzymatic synthesis of a wide variety of glycolipids can be achieved using a sustainable process and renewable substrates (sugars, choline chloride, fatty acid…). The key-point of the described DES-based solvent system is that it acts simultaneously as substrate and solvent for the synthesis of glycolipids and alditollipids. The versatile biocatalyst iCalB was successfully used to carry this enzymatic conversion. It was also demonstrated that this immobilized lipase is not specific for one glycolipid product in this process. Indeed, besides sugar mono-laurates many poly-acylated sugars were formed during the synthesis.

In addition to these interesting features concerning a sustainable glycolipid production, renewable resources can also be incorporated to the process. Indeed, the use of lignocellulose, the most abundant renewable resource of the world, as substrate has been demonstrated [79]. To be processed further, lignocellulose (carbohydrates polymer) has been pretreated to produce sugar monomers. These sugars from lignocellulose can therefore be used as starting material for the enzymatic synthesis of surface-active agents. After purification and drying, the resulting glucose- and xylose-rich fractions were used successfully to create a DES and subsequently produce glycolipids by the previously described process. Furthermore, forming a DES with these sugars is an elegant way to overcome the problem of low solubility of sugars in other water-free solvents. In such eutectic type of solvent, the sugars are easily available for the reaction in the solvent because they compose most of the latter.

Renewability of the sugar moiety production is a good step toward achieving a sustainable glycolipid synthesis. However, as described by Siebenhaller *et al*., an eco-friendly process for the hydrophobic tail of the surfactant as well is something to be taken care of. In this case, the hydrophobic tail of a glycolipid ester is a fatty acid [86]. To achieve such aim, the work described here managed to harness beechwood components in three different ways: as a sugar component, as part of the DES and finally as carbon source for the microbial production of the fatty acid component. In the detailed proof of principle, fatty acids were gained from single cell oil produced by the oleaginous yeast *Cryptococus curvatus* cultivated with cellulose fiber hydrolysate as carbon source. The newly bio-synthetized fatty acids are allowed then to undergo an enzymatic esterification with the also eco-based sugars inside the DES media. Such successful approach permits a 100% based lignocellulosic biomass sugar ester production.

1.8. β-Glucosidases in Glycolipid Production

Glycolipid synthesis using lipases is widely established and offers undeniable advantages over chemical synthesis; however, two aspects must be considered: (I) Lipases connect sugar moiety and alkyl residue by an ester bond. (II) Ester bonds are highly reactive with acids, bases, active metals, oxidizing agents and reducing agents. Therefore, they are easily hydrolyzed making them relatively unstable. This can be an advantage in terms of degradability but might also restrict the application of the glycolipid. In contrast, ether bonds are less reactive, and therefore, much more stable (**Figure 3**). As outlined above, lipases produce regiospecifically a main product, but also non-specifically several other by-products which is thought to be avoided when choosing enzymatic over chemical synthesis.

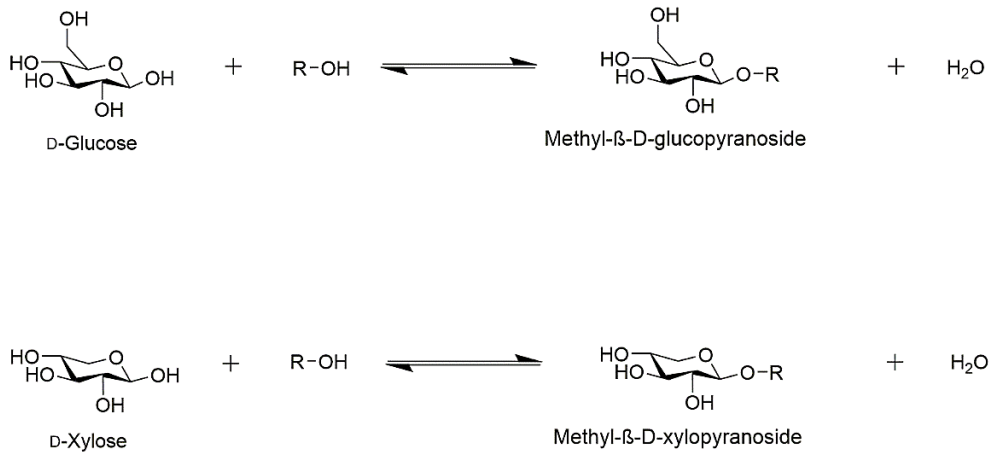


Figure 3. Glycolipid formation by almond β-glucosidases *via* reverse hydrolysis route

Regarding the enzymatical conversion of glycolipids, β-glucosidases are an alternative to lipases. β-Glucosidases (E.C.3.2.1.X) are co-factor independent hydrolases which naturally cleave glycosidic bonds in polysaccharides. Similarly, to lipases, glucosidases can also catalyze the formation of glycosides and display a broad range of substrates. They operate at pH 7, 50°C, atmospheric pressure and are highly stereo- and regiospecific providing a controlled stereochemistry toward the anomeric center of the newly formed glycosidic bond [87–90]. Furthermore, numerous and low-priced β-glucosidases are available on the market.

Table 2. Synthesis of glycolipids by almond ß-glucosidase *via* reverse hydrolysis route using different glycosyl donors and acceptors.

Source of enzyme	Glycosyl donor	Gylcosyl acceptor	Product	Yield (%)	Time (h)	Ref
Almond β-glucosidase	Xylose	Methanol	Methyl-ß-D-xylopyranoside	Poor Yields	6 days	[26]
Almond β -glucosidase	Xylose	Ethanol	Ethyl-ß-D-xylopyranoside	Poor Yields	6 days	[26]
Almond β -glucosidase	Xylose	Propanol	Propyl-ß-D-xylopyranoside	Poor Yields	6 days	[26]
Almond β -glucosidase	Xylose	Butanol	Butyl-ß-D-xylopyranoside	Poor Yields	6 days	[26]
Almond β -glucosidase	Glucose	Hexanol	Hexyl-ß-D-glucopyranoside	11	144h	[91]
Almond β -glucosidase	Glucose	Methanol	Methyl-ß-D-glucopyranoside	58	54	[92]
Almond β -glucosidase	Glucose	Butanol	Butyl-ß-D-glucopyranoside	30	Not given	[93]
Almond β -glucosidase	Glucose	Octanol	Octyl-ß-D-glucopyranoside	20	200	[94]

Synthesis of APGs by glucosidases can be achieved by two strategies: either by the reverse hydrolysis pathway (thermodynamically controlled) or by the transglycosylation pathway (kinetically controlled). In the first pathway β-glucosidase uses a monosaccharide and a fatty

alcohol to form an APG (**Figure 4**). Therefore, it is important to shift the reaction equilibrium towards the synthesis reaction by reducing the water content in the reaction mixture and by increasing the substrate concentration [95,96]. For the second pathway, activated glycoside donors are used together with fatty alcohols to form an alkyl glucoside *via* a glycosidic bond. In earlier studies, in which β-glucosidase was used as a biocatalyst, it was shown that 3 different ethoxylated glycosides could be produced in supersaturated solutions of glucose or *p*-nitrophenyl β-D-xyloside [97]. Furthermore, it was shown that it is also possible to use β-glucosidase in a biphasic system of buffer and *n*-alcohol [98,99] to produce *n*-alkylglycosides. In 1999, Yi and Vulfson demonstrated a new approach in which a β-glucosidase, previously microencapsulated in a polymer, was used in an aqueous-organic two-phase system to produce alkyl-β-D-glucosides (**Table 2**) [100].

Using almond β-glucosidase, various sugars were successfully used as substrates for the synthesis of APGs: fructose, mannose, xylose, rhamnose as well as trehalose and galactose. In addition, various fatty alcohols such as butanol, hexanol, octanol and decanol as alkyl chain were accepted by the enzyme (unpublished results).

Especially, the resulting glycolipid from the disaccharide trehalose could have very interesting properties due to the sugar moiety. Trehalose lipids have been reported to be less toxic than synthetic surfactants [101] and feature several biological properties like antimicrobial, antiviral [102], and antitumor activities [101,103,104]. They also can act as therapeutic agents due to their interaction with cell membranes [102,105]. By using β-glucosidase, the reaction hardly produces any by-products, and the first results indicate a regioselective synthesis, which results in very pure products (unpublished results).

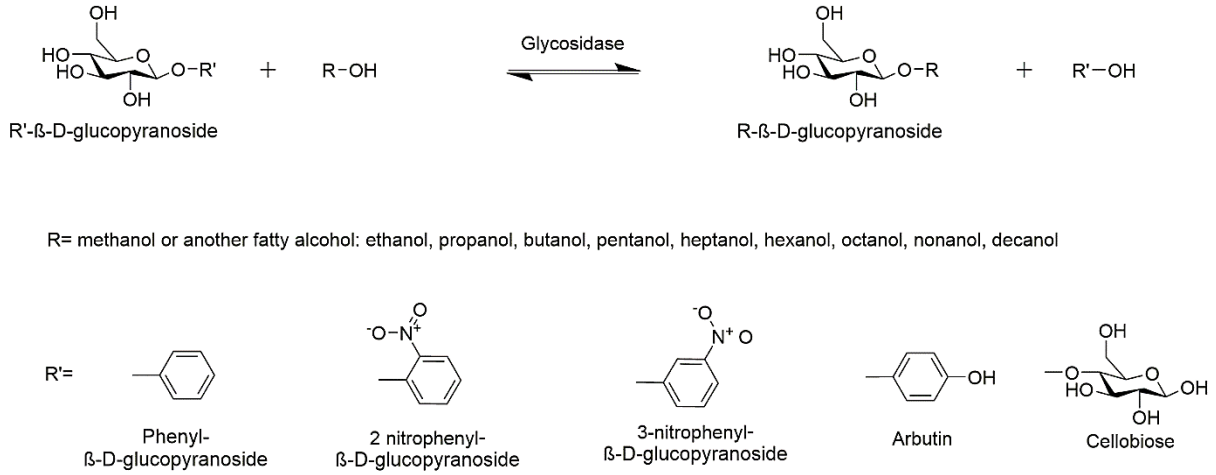


Figure 4. Activated glycoside donors and common n-alcohols (= nucleophilic acceptor) used for enzymatic synthesis to generate new glycolipids with β-glucosidases.

1.9. Economical Aspect of Glycolipid Production

In 2011, the chemical industry produced around 14 million tonnes of surfactants with annual sales of around 23 billion US dollars (Transparency Market Research, 2012). With the background of uncertainty and variability in oil prices, consumer demand for environmentally friendly chemicals is increasing and greater attention is being paid to the production and use of surfactants from biomass. In Europe, for example, the annual growth rate of biobased surfactants was 3% between 2008 and 2013, with APGs showing the most significant growth. Global demand for APGs has induced the largest producers of APGs to increase their output

volumes. A detailed cost analysis (LCA) of the current glycolipid production pathway would go beyond the scope of a theoretical background [106,107], so this chapter will give a brief overview of the general process. To compare the different technologies, the disadvantages of Fischer glycosylation are explained and the possible advantages of synthesis using a biocatalyst are described.

The production processes for the most relevant glycolipids on the market can be divided into 5 steps: 1. Raw material acquisition, 2. Logistics (2.1 Farm to pellet plant, 2.2 Pellet plant to refinery, 2.3 product transportation), 3. Feedstock processing, 4. Biorefining: chemical processing phase and 5. Storage. An exemplary chemical process results in a market price of $1.60-$3.20 per kg for APGs produced from palm kernel oil and wheat grain. This price is calculated as follows: the biomass cultivation per kg APG costs 0.06$ and labour costs account for 0.33$ per kg glycolipid. Packaging and storage amount to 0.30$ per kg of product, pre-processing and refining is at 0.71$ per kg (0.64 $ + 0.07 $) and the transportation costs are 0.52$. Using this calculation total costs of 1.92$ per kg are obtained. The share of pre-processing and refinery is 0.71$ and thus makes up a large part of the total costs.

On the industrial scale, APGs are produced by Fischer glycosylation, a process which requires precise parameters and elaborated techniques. The first alkyl glycoside was synthesized from glucose and identified in the laboratory by Emil Fischer (1893). The common catalysts used for Fischer glycosylation are sulphuric acid, hydrochloric acid, or phosphoric acid. At the end of the process, the acid catalyst is neutralized e.g., with sodium hydroxide. APGs produced by Fischer glycosylation are complex mixtures of α/β anomers and pyranoside/furanoside isomers of glycosides and polyglycosides. The ultimate composition depends on the saccharide type and results from the competition of many reactions. In addition, two sugar units can be covalently linked in different ways for APG oligoglycosides. A diglycoside can therefore have approximately 30 isomeric forms; this number increases to several hundred for triglycosides and to several thousand for tetraglucosides. Most catalysts lead to the formation of saccharide polymers (glucose turns into polydextrose), which leads to a high viscosity of the product mixture, even at low concentrations, resulting in product loss during the separation and purification steps [108]. Thermodynamically preferred polyglycol formation can be minimized by using large amounts of alcohol during glycosylation, but this reduces productivity and increases operational costs. Other disadvantages of the process are the use of high amounts of corrosive or toxic acidic catalysts and the need of careful control of reaction pressure to limit the formation of polymerized sugar. The use of high volumes of alcohol (an economic decision criterion due to increased alcohol costs) and high temperatures is necessary to reduce the reaction time which finally leads to by-products [109]. Due to the low solubility of carbohydrates in long fatty alcohols, the reaction requires a complicated intermediate glycosylation step with an alcohol such as methanol when using long-chain fatty alcohols. Solvents and the use of ionic liquids have been discussed, but they do not allow cost-effective surfactant production as the separation of tensides becomes more difficult. The immiscibility of sugars with fatty alcohols is one of the major obstacles to produce APGs with high yield and productivity. Compared to the chemical process (described in the previous subsection), a biotechnological process in DES with a technical enzyme as catalyst would have some advantages. No acids are needed as catalysts, the process temperature can be reduced from 80°C to 50°C and overpressure is not required. Therefore, less energy would be needed, and products would not be coloured eliminating a bleaching step after the process. In addition, less alcohol must be used, which is, as mentioned above, an important issue because fatty

alcohols are expensive. These advantages might compensate higher costs for enzymes as catalysts and/or lower yields.

1.10. Conclusions

Glycolipids are a very versatile class of ecological biosurfactants exhibiting excellent foaming and wetting abilities and show often antimicrobial activity. By using enzymatic synthesis, a nearly unlimited number of novel glycolipids can be produced far beyond the natural diversity. In contrast to chemical synthesis, anomerically pure products can be achieved without the need of using protecting groups. Furthermore, enzymatic synthesis is in accordance with the principles of green chemistry, especially if the solvent system is chosen wisely. Lipase-catalyzed syntheses in organic solvents are established processes achieving high yields. However, to ensure sustainability also other solvents should be explored. Deep eutectic solvents offer a cheap and eco-friendly alternative, but synthesis yields are still low and lack economic competitiveness making increased research efforts necessary. In addition, β-Glucosidases have not been studied much regarding glycolipid synthesis, although they have two big advantages over lipases: they form highly stable ether bonds and exhibit strict regioselectivity while also accepting a wide range of sugars and fatty alcohols as substrates, while being cheap to purchase as they are manufactured on industrial scale. Therefore, β-glucosidases can diversify the pool of industrial relevant enzymes beyond Novozym 435.
Synthesis of tailor-made glycolipids is a prerequisite to elucidate structure-activity-relationships enabling rational design of surfactants. By using enzymes, functional relationship of the different bond types (ester/ether) can be studied, or double bonds can be introduced into the alkyl chain to investigate their influence on the CMC value. This would allow the selection of the best performing structures to improve the surfactants´ properties, so less glycolipid may be needed to obtain formulations of equivalent performances.

1.11. Research Proposal

As the demand for eco-friendly surface-active agents is increasing worldwide, we aimed at producing bio-based and biodegradable glycolipids using innovative and integrated processes. Sorbitol laurate, among other sugar or sugar-alcohol esters, was chosen as model for the production of potential competitive candidates against non-green and petroleum derived surfactants. In regard to current concerns about sustainably, the processes are revolving around heterogenous biocatalysis, designed to be intrinsically benign, through the use of lipases which are major assets for renewable synthesis. Thus, we used the most straightforward way to obtain glycolipids *via* transesterification between a sugar alcohol and, in a first time, a vinylated fatty acid chain, in low water activity/content media. Moreover, to follow green chemistry principles one must preclude or at least reduce the use of volatile solvents. In consequence, usage of Deep Eutectic Solvents (DES), a liquid phase made of ubiquitous primary metabolites, made sense not only to dissolve lipophilic substrates but also to facilitate downstream processing, enzyme recovery and waste-management.

In this scope, an elegant and innovative reaction system in which, the sugar alcohol is a substrate and also a part of the solvent, has been tuned. By modifying different parameters of this system such as the water content, biocatalyst and substrate concentrations, a first step into optimization was made .

The screened enzymes, and all the different forms of immobilized enzymes, were commercially available as a requirement. The efficacies of the different enzymes were compared according to the yields of glycolipid production as quantifications were owed to a newly developed HPLC-ELSD analytic method.

In addition, purification and characterization strategy by means of thin-layer chromatography (TLC), medium pressure preparative liquid chromatography (MPLC), high-performance chromatography (HPLC), nuclear magnetic resonance (NMR) and mass spectrometry (MS) were necessary to assess chemical structures in a robust and reliable way.

Different types of reactors have been tested as well such as batch a Stirred Tank Reactors (STR), proofing the scalability of our process and allowing preparative scale production of surfactants. Microwave technology was scrutinized as well, and due to its alternative heating method, the process was intensified in various, quasi water-free, media. The recovery and re-use of the biocatalyst were investigated alongside the extraction efficiency that is part of the downstream processing.

Finally, the bioactivity of DES-like honey mixtures enriched with glycolipids synthetized *in situ*, were tested and the observed activity was characterized *via* respectively agar-well assay and a designed biosensing experiment. We gave thereby, strong evidence of the potential that sugar-saturated mixtures and glycolipids entail for pharmaceutical applications as well.

2. Lipase-Catalyzed Production of Sorbitol Laurate in a “2-in-1” Deep Eutectic System: Factors Affecting the Synthesis and Scalability

This chapter is partly based on the publication:

Lipase-Catalyzed Production of Sorbitol Laurate in a “2-in-1” Deep Eutectic System: Factors Affecting the Synthesis and Scalability
André Delavault, Oleksandra Opochenska, Laura Laneque, Hannah Soergel, Claudia Muhle-Goll, Katrin Ochsenreither and Christoph Syldatk
Molecules, 2021, 26(9), 2759.
doi: 10.3390/molecules26092759
Published: 07.05.2021

Authors contributions:

André Delavault: conceptualization, methodology, formal analysis, investigation, writing of the original draft, writing of the reviews, and editing

Oleksandra Opochenska: methodology, formal analysis, and investigation

Laura Laneque: formal analysis, and investigation

Hannah Soergel: methodology, formal analysis, investigation, writing of the original draft, writing of the reviews, and editing

Claudia Muhle-Goll: methodology, formal analysis, investigation, writing of the original draft, writing of the reviews, editing and supervision

Katrin Ochsenreither: writing of the reviews, editing and supervision

Christoph Syldatk: supervision and funding acquisition

2.1. Introduction

Glycolipids and more generally surfactants are getting an increasing attention and are currently under scrutiny from the scientific community because of their various fields of applications [110]. This versatility allows them to fill different functions such as excipient in drugs, encapsulating agent, lubricants and many more [111]. Such quasi-omnipresence makes their use valuable in very concrete and industrialized applications such as oil recovery enhancement or even as dough conditioning [112,113]. Moreover, the recent sanitary crisis, that resulted into the current global pandemic, is announced to increase further this production, as surfactants are among the main components present in hygiene products [114]. Thus, the whole chain of production starting from the acquisition of raw materials to the formulation and storage should be logically stimulated as well. Given the context, it appears topical to develop processes that enable safe, reliable, and straightforward production of such compounds. In addition, the processes and the resulting compounds should respond to present concerns in relation with sustainability, renewability, and biodegradability. Such problems brought to awareness and theorized by the Green Chemistry field, revolve around the "benign by design" concept [115]. By definition, it means that chemical products and processes should be designed to eliminate the generation of hazardous substances [116]. Therefore, it reduces the number of steps necessary for the production, from the synthesis to the downstream processing (DSP), and simplifies the overall process chain.

Glycolipids such as sugar esters (SEs) are, in this regard, relevant candidates that meet the requirements, raising in the meantime, several challenges and problems that are tackled in this work.

The well-established chemical production of SEs presents several disadvantages and limitations such as low specificity, low selectivity and use of corrosive reactants [117]. Consequently, alternative ways were pursued to prevent the use of organic solvents, as they represent most of the waste in industrial processes and syntheses [118]. Moreover, they present undeniable limitations for the solubilization of polyols such as sugars. In this aim, production of SEs in ionic liquids (ILs) [48] and in Deep Eutectic Systems (DESs) [119] were investigated. The latter, described first by Abbott *et al*., represent a cheaper, less toxic, and facile option among the low-transition temperature mixtures [120]. Additionally, their low water contents and low water activities lead lipases to reverse their activity and form ester bonds using relatively mild and harmless conditions [121].

Numerous applications of DESs in environmentally friendly chemical processes have been made, notably for the production of pharmaceutically relevant building blocks and scaffolds [122–124]. Moreover, DESs can also be used for the extraction of valuable compounds such as phenolics contained in olive oil wastes [125], adding them as a sustainable tool for food waste treatment and re-valorization. In our similar and inherently benign approach, sorbitol is simultaneously part of the solvent as a DES partner, in combination with choline chloride, and part of the lipase-catalyzed reaction as a substrate. Intrinsically, this reaction design solves solubility problems of the polyols while saturating the system with the acyl acceptor. This "2-in-1" principle described first by Siebenhaller *et al*. [126], using notably C4 to C6 sugars in combination with vinyl fatty esters, presented however exceptionally low yields (~4%) and lacked proof of scalability [127]. The use of vinyl esters for SE synthesis described by Bornscheuer *et al*. is thermodynamically favored as the vinyl adduct is, during the reaction, converted to the side-product ethenol which itself tautomerizes in the highly volatile

acetaldehyde [128] (Figure 1). For the first time, we enhanced further the conversion yield of this advantageous system, using a relatively better acyl acceptor for the reaction [129]. Indeed, sorbitol and more generally sugar alcohols, showed successful use in prior research as they are also less sensible to degradation than their actual sugar analogues [130–133]. Moreover, laurate monoesters are valuable for many recently developed applications such as drug encapsulation [134].

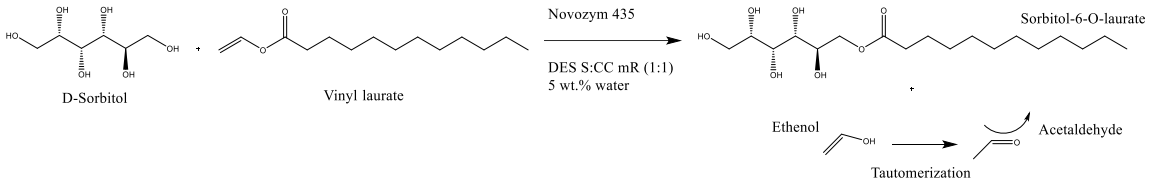


Figure 5. Lipase-catalyzed transesterification reaction between D-sorbitol and vinyl laurate. Evaporation of highly volatile acetaldehyde makes the conversion irreversible. S: Sorbitol; CC: Choline Chloride; mR: molar Ratio.

We initiated, herein, the optimization of the "2-in-1" process by selecting Novozym 435® as a suitable biocatalyst among various commercially available lipase formulations and sought, specifically to our system, its optimal performing parameters. We varied parameters (One-Factor-at-a-Time, OFaaT) one at a time, such as biocatalyst concentration, time of reaction, substrate concentration and water content. The optimal temperature for the use of this specific lipase has been extensively studied in literature. Moreover, previous work from Siebenhaller *et al*. [135] and Hollenbach *et al*. [136] presented concomitantly, that in the range of 50-60°C the enzyme meets its optimal activation energy

In addition, we present robust purification and identification strategies promoted by suitable analytical but also flash-chromatography methods for the quantification and preparative scale (>500 g) production of sorbitol-6-*O*-laurate using a batch stirred tank reactor (STR). The successful investigation was insured using relevant Evaporative Light Scattering Detection (ELSD) combined with HPLC and flash-chromatography. Thus, the production of pure standards enabled the establishment of the calibration curve and the 1D-, 2D-Nuclear Magnetic Resonance (NMR) and Mass Spectrometry (MS) characterization of selectively-acylated sorbitol monolaurate.

2.2. Materials and Methods

2.2.1. Materials

Vinyl laurate was purchased from Tokyo Chemical Industry Co., Ltd. (TCI Europe, Belgium). Lipase formulations: Novozym 435®, Lipozyme 435® and Novozym NS 81356 were given by Novozymes (Denmark). CalB Immo Plus® was given by c-Lecta (Germany). CalA Immo 150, Lipase TL CLEA, Lipase CA CLEA and all other chemicals were purchased from either Carl Roth GbmH & Co. KG (Karlsruhe, Germany) or Sigma Aldrich Chemie GmbH (Taufkirchen, Germany) if not stated otherwise. The other 9 lipase formulations were acquired in the Novozymes Lipase Screening Kit purchased from Strem Chemicals (Newburyport, MA, USA).

2.2.2. DES Preparation and Standard SL Synthesis for Enzyme Formulation Screening

The sorbitol and choline chloride-based DES, dubbed "Sorbit DES", was prepared and validated according to the procedure described by Dai *et al*. and Hayyan *et al*. [137,138]. The

water content was varied (1.25, 2.5, 5, 7.5 and 10 wt.%) then controlled according to method described in subsection 2.2.6.

In a 5 mL Eppendorf tube, were introduced subsequently 1.5 mL of warm Sorbit DES, vinyl laurate (195 µL, 170 mg, 0.75 mmol, 0.5 M) and 30 mg of enzyme formulation (20 g/L) (Table 1). The tubes containing the reaction mixture were agitated in a rotator and vortex mixer (program U2) from neoLab (Heidelberg, Germany) at 90 rpm and 50°C. To get a triplicate for each measure, three tubes were collected for each time point at : 0.5h, 4h, 8h, 24h , 28h, 32h, 48h, 72h and 96h. The latter were then processed for further analysis as described in subsection 2.2.8., the conversion yields were calculated as the percentage of molar ratio of sorbitol monoester produced to the total amount of vinyl laurate added to the reaction system.

2.2.3. Enzyme Formulations Screening

To compare the lipase formulations (**Table 4**) between them, an identical concentration of the latter was used each time (20 g/L) and same substrate concentration (0.5 M vinyl laurate) was provided to the media at 50°C. For this comparison, the reaction was stopped after 48h.

2.2.4. Influence of Enzyme Concentration

To examine the optimal concentration of lipase formulation, different concentration of Novozym 435® (10, 20, 30, 40, 50 and 60 g/L) were tested without varying any other reaction parameter.

2.2.5. Optimization of Vinyl Laurate Amount

To address the optimal vinyl fatty ester concentration for the reaction, different vinyl laurate concentrations (0.25, 0.5, 0.75, 1 and 1.25 M) were tested. All other reaction conditions were kept constant.

2.2.6. Water Content, Viscosity and Water Activity Analysis

The water content was assessed with Karl-Fischer titration using a TritoLine 7500 KF trace from SI Analytics (Mainz, Germany) at 20°C in combination with Aquastar CombiCoulomat fritless (Merck Millipore, Darmstadt, Germany) as analyte. Water standards of 0.1% and 1% in xylene from Merck Millipore (Darmstadt, Germany) were used to test titrator's accuracy before the measurements.

The viscosity was measured at 50°C with a viscosimeter MCR 501 using a CC10 concentric cylinder (Anton-Paar, Graz, Austria) with about 1 mL of liquid for each water content for the Sorbit DES.

The water activity was measured at 50°C with a LabMaster-aw neo $A_w$meter (Novasina, Lachen, Switzerland) using 3 mL of liquid for each water content for the Sorbit DES.

2.2.7. Scale-up Procedure and Downstream Processing for Standard and Bulk SL Production

In a 2.5 L Minifors bioreactor (Infors HT, Bottmingen, Switzerland) were introduced 500 mL of warm Sorbit DES, prepared as described in subsection 2.2.2. Medium temperature was first equilibrated to 50°C then subsequently were introduced 25 g of Novozym 435® (50 g/L) and vinyl laurate (65 mL, 56.62 g, 0.25 mol, 0.5 M). The reaction mixture was stirred at 300 rpm with a single three-bladed spiral propeller (D = 54 x 12 mm) (Infors HT, Bottmingen,

Switzerland). After 48h, the reaction was stopped, the media diluted with 500 mL of double-distilled water and filtrated through a Büchner funnel. 200 mL of brine were incorporated to the aqueous phase that was then extracted 6 times with a 1:1 volume ratio of ethyl acetate. The organic phases were gathered and chemically dried over $MgSO_4$ before being dry evaporated with a rotative evaporator.

For the purification of SL standard that was used for the calibration curve, 2 g of the crude paste was re-dissolved in chloroform to be adsorbed over 4 g of Celite 545 for flash chromatography purification using the solid loading method. To purify this crude, a Reveleris PREP purification system equipped with a 12 g Chromabond® Flash RS 15 Sphere SiOH column (Macherey-Nagel, Düren, Germany) was used. Elution solvents were chloroform and methanol with a gradient such as: 2nd solvent percentage started at 0% for 1.5 min, 7% for 9.5 min, 15% for 3 min, and finally 100% for 3 min. The second fraction, containing SL, was collected at 7.5 min. The latter was dry evaporated on a rotary evaporator for further use and analysis. ~0.75 g of dried, white powder. (**Appendix: Figure A1**)

For bulk production of SL, the entire crude (~25 g) was re-dissolved in the necessary minimal volume of ethyl acetate (~400 mL) and washed with 1 × 400 mL of brine then 3 × 400 mL of double distilled water to remove unreacted sorbitol. The organic phase was dried over $MgSO_4$ and dry evaporated on a rotary evaporator. ~10g of dried, white powder. (**Appendix: Figure A2**)

2.2.8. Sample Preparation and HPLC-ELSD Quantification Method

Tubes produced by the methods described in 2.2.2.—2.2.5. were prepared and analyzed as follows. For extraction of the glycolipid and its quantification with HPLC-ELSD the following procedure was applied. 1 mL of double distilled water was added to the tube containing the reaction mixture and vortexed for 45 s. 2.5 mL of ethyl acetate were then added to the warm solubilized DES, subsequently the extraction took place at 50°C for 20 minutes with use of the orbital shaker set on program U2 and 90 rpm (neoLab, Heidelberg, Germany). 1 mL of the upper phase was then aliquoted and dried on a centrifugal evaporator, to be resolubilized in 1 mL of chloroform/methanol (75:25 v/v) and 100 μL were placed into a HPLC vial for further quantification.

The method described by Hollenbach *et al*. [136,139] was used with slight modifications as follows. Kinetex EVO C18 (2.6 mm, 250 mm x 4.6 mm) column from Phenomenex (Aschaffenburg, Germany) with an accompanying guard column (4 mm x 3.0 mm ID) of the same phase, using an Agilent 1260 series liquid chromatograph (Waldbronn, Germany) equipped with a quaternary pump, an autosampler and a column oven. An evaporative light scattering detector (ELSD) from BÜCHI Labortechnik (Essen, Germany) was used for detection. The mobile phase, solvent A, was water and solvent B was acetonitrile. The flow rate was 1 mL/min, and a gradient was used for separation of product and substrates: starting from 40% A-60% B, then 0-10 min a linear gradient up to 35% A-65% B, followed by another linear gradient from 10 to 15 min up to 25% A-75% B. This gradient was held for 5 min, followed by a reconditioning step of the column with 40% A-60% B for 5 min. The injection volume was set to 10 μL. The column was operated at 50°C. The detector was operated at 38°C with a gas flow (air) of 1.5 mL/min. The gain was set to 1. The retention times were 2.1 min for sorbitol and 3.5 min for SL and ~9.5 min for lauric acid. (**Appendix: Figure A3**)

$$\text{Yield } [\%] = \frac{\text{nSL [mol]}}{\text{nVinyl laurate [mol]}} \times 100\%. \quad (1)$$

$$\text{Specific reaction velocity } [\mu\text{mol/h/g}] = \frac{\text{nSL [}\mu\text{mol]}}{\text{mBiocatalyst [g]} \times \text{time [h]}}. \quad (2)$$

n: number of moles

m: mass

2.2.9. Spectroscopic and Spectrometric Methods for Structural Elucidation of SL

For nuclear magnetic resonance (NMR) spectroscopy, 10 mg of purified SL was dissolved in 0.6 ml CD_2Cl_2/d6-acetone (4:1, by vol.). 1D ^{1}H NMR spectroscopy and 2D ^{1}H-^{1}H correlation spectroscopy (COSY), ^{1}H-^{1}H total correlation spectroscopy (TOCSY), ^{1}H-^{13}C heteronuclear single-quantum correlation (HSQC) spectroscopy and ^{1}H-^{13}C heteronuclear multiple-bond correlation (HMBC) spectroscopy were performed on a Bruker AVANCE III 600 MHz spectrometer (Bruker BioSpin, Rheinstetten, Germany) equipped with a TCI cryoprobe at a temperature of 27 °C. Spectra were processed and analyzed using Topspin 4.0.1 (Bruker BioSpin) and MestReNova 14.2.0 (Mestrelab Research S.L., Santiago de Compostela, Spain). Chemical shifts were referenced to the ^{1}H and ^{13}C resonance of tetramethylsilane (TMS).

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

2.2.10. Data Treatment and Statistical Analysis

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

2.3. Results

In the following sections we report the investigation of commercially available lipase formulations to produce SL alongside a newly developed quantification method that allowed the investigation of the impact of several synthesis factors. Following the product formation over time also allowed optimization of reaction time, enzyme, and substrate concentration and subsequently, water content of the DES. Finally, the titer of the optimized process as well as a proof of scalability and technical notes for the use of a batch STR followed by the DSP procedure are presented.

2.3.1. Sorbitol Monolaurate (SL) Quantification

We herein successfully separated and quantified SL using a newly developed analytical HPLC-ESLD method which allowed the differentiation of the products and substrates (**Appendix: Figure A3**). Sorbitol had a retention time of 2.1 min, 3.1 min for SL and ~9.5 min for lauric acid (not integrated). Due to the low baseline noise and suitable peak resolution (**Table 3**), it was possible to quantify SL in a range between 0.75 g/L and 30 g/L with use of

two linear ranges of calibration as ELSD does not provide a linear response when analyte concentration increased.

Table 3. Chromatographic and analytical characteristics of SL analysis using HPLC-ESLD.

Retention time (SL)*	**3.55 — 3.59 min**
Peak width**	0.060 — 0.091 min
$Resolution_{sorbitol - SL}$ (n = 3)	17.5
$Resolution_{SL - lauric\ acid}$ (n = 3)	7.6
Baseline noise (n =3)	0.22 ± 0.07 mV
Limit of detection (signal/noise = 3)	< 0.04 g/L
Limit of quantification (signal/noise = 10)	0.04 g/L
1st range of linear calibration	
Correlation coefficient (R^2, n = 3)	0.9967
Equation of linear calibration	y = 0.001x + 0.988
Linear range of calibration	0.75 — 15 g/L
2nd range of linear calibration	
Correlation coefficient (R^2, n = 3)	0.9993
Equation of linear calibration	y = 0.002x - 21.639
Linear range of calibration	20 — 30 g/L

Note. *Inter-day variance of retention time measured at 3 different days. ** Concentration 0.75 — 30 g/L.

2.3.2. Commercially Available Formulations Screening

Figure 6 shows that highest yields of vinyl laurate conversion into SL were obtained by Lipase B from *Candida antarctica* (CA) formulations such as Novozym 435®, Lipozyme 435® and Lipozyme CALB L (liquid formulation). Regardless of the applied form, all of them seem to reach statistically equivalent results of ~20% under non-optimized reaction conditions. However, it is important to note that liquid formulations potentially contain more catalytic material than the immobilized ones, showing the limitations of our comparison.

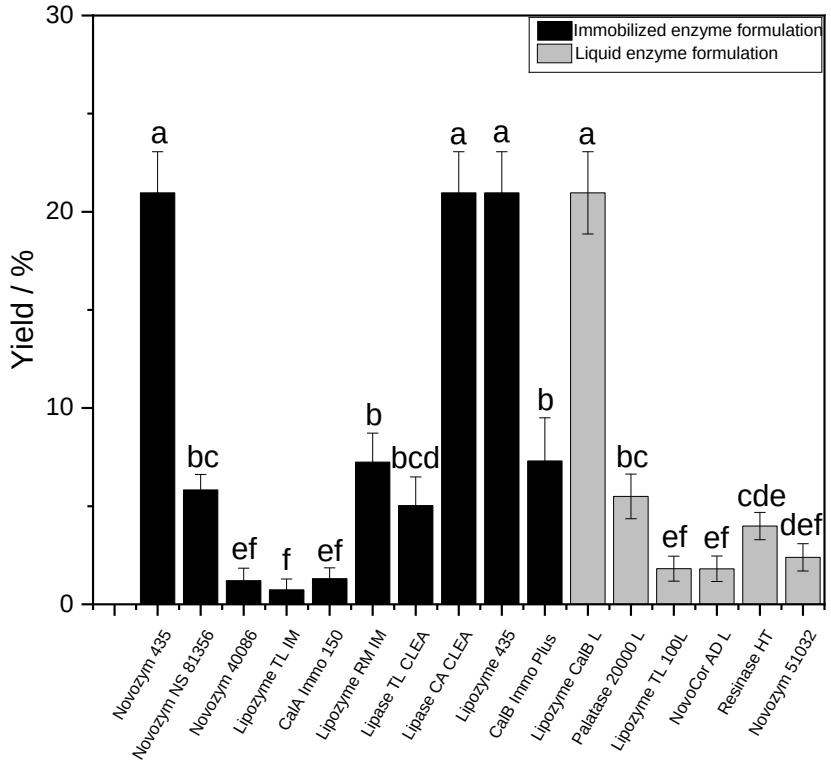


Figure 6. Comparison of SL conversion yields calculated from 0.5 M vinyl laurate. A triplicate was done for each screened commercially available formulation after 48 h at 50°C. a, b, c, d, e and f show statistically significant differences (p<0.05).

As we compare these lipase formulations based on applied concentrations (20 g/L), the quantity of actual enzyme present in the reaction most likely differs, moreover, very little data on the matter are made available by the producers. The quantity of enzyme is however directly linked to the amount of formulation used, thus when comparing immobilized enzyme this variable is intrinsically highlighted. As a result, we are, among other factors, comparing qualitatively the immobilization efficiency of the various formulations tested. In this aim and in our system, either using the food grade formulation (Lipozyme 435®) or the technical grade one (Novozym 435®) did not lead to significant differences in titer after 48 hours of reaction (**Table 4**). Interestingly the Cross-Linked Enzyme Aggregate (CLEA) from CA seemed to reach statistically similar levels of performance. Therefore, in the following sections, we chose to report the influence of several factors affecting our system. For this purpose, we used Novozym 435® as the archetype of the commercially available lipase formulation for SE synthesis. As a first step, we decided to investigate the evolution of SL titer over time.

Table 4. Titer (g/L) of SL obtained after 48 hours with commercially available enzyme formulations.

Formulation name	Reported activity*	Formulation type	Reported optimal temperature range (°C)*	Titer (g/L)**
CalA Immo 150	500 U/g	Immobilized	N.C.	2.3 ± 0.9
CalB Immo Plus	>9000 PLU/g	Immobilized	60-80	13.3 ± 3.0
Lipase CA CLEA	>1.5 U/mg	Cross-linked	>40	38.2 ± 3.8
Lipase TL CLEA	≥25 U/mg	Cross-linked	40-60	9.2 ± 2.7
Lipozym 435	9000 PLU/g	Immobilized	N.C.	38.2 ± 3.8
Lipozyme CALB L	5000 LU/g	Liquid	30-60	38.2 ± 3.8
Lipozyme RM IM	275 IUN/g	Immobilized	30-50	13.2 ± 2.7
Lipozyme TL 100L	100 KLU/g	Liquid	20-50	3.3 ± 1.2
Lipozyme TL IM	250 IUN/g	Immobilized	50-75	1.3 ± 1.0
NovoCor AD L	6000 LU/g	Liquid	30-60	3.3 ± 1.2
Novozym 40086	275 IUN/g	Immobilized	30-50	2.2 ± 1.1
Novozym 435	10000 PLU/g	Immobilized	30-60	38.2 ± 3.8
Novozym 51032	15 KLU/g	Liquid	35-70	4.4 ± 1.3
Novozym NS 81356	N.C.	Immobilized	N.C.	10.6 ± 1.4
Palatase 20000 L	20000 LU/g	Liquid	30-50	10.0 ± 2.1
Resinase HT	50 KLU/g	Liquid	≤90	7.3 ± 1.3

Note. N.C.: Not Communicated. * Reported data are provided by the producers and are available online. ** Experiments were performed as triplicates under identical conditions: 0.5 M of vinyl laurate, 20 mg of formulation in 1.5 mL of Sorbit DES (sorbitol/choline chloride, 1:1, mR, 5 wt.% water) after 48h at 50°C and data is presented as mean values ± standard deviations (n = 3, p-value < 0.05).

2.3.3. Product Formation Over Time

To determine the optimal reaction time to produce the maximum amount of SL, concentration of produced glycolipid was recorded over 96 h. Thus, we reached a saturation of product after 48 h with apparently no significant changes in concentration, even up to 96 h of reaction as shown in **Figure 7**.

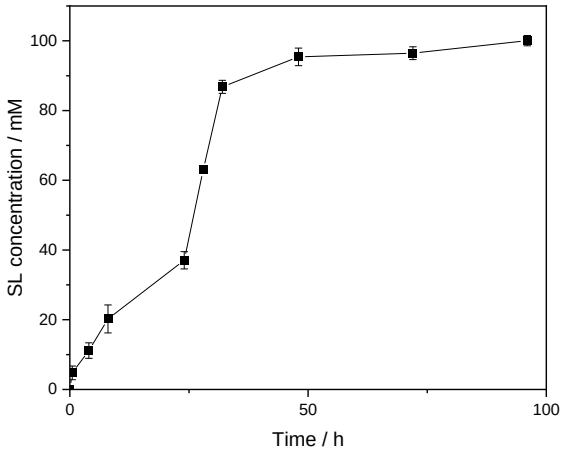


Figure 7. Time course of the reaction under unoptimized conditions: 0.5 M of vinyl laurate, 20 mg of Novozym 435®, and 1.5 mL of Sorbit DES as solvent (sorbitol/choline chloride, 1:1, mR, 5 wt.% water) at 50°C.

Under these unoptimized conditions using Novozym 435®, a concentration of 95 ± 2.5 mM of product was obtained after 48 h of reaction, which translates to a specific productivity of 100 µmol/h/g. Until now, no data has been reported regarding the use of a "2-in-1" DES system for lipase-catalyzed transesterification between a vinyl laurate and sorbitol to synthetize such monoacylated sugar alcohol ester.

2.3.4. Effect of Enzyme, Substrate and Water

Herein, Novozym 435® was used as the biocatalyst for the reaction that was carried out in the "2-in-1" Sorbit DES. Factors impacting the reaction (i.e., enzyme concentration, enzyme dosage and water content in the media) have been investigated to find the optimal parameters for each condition. From **Figure 8**, it can be discerned that the optimal values were 50 g/L enzyme, 0.5 M vinyl laurate and 5 wt.% water.

Table 5. Averaged values of viscosity and water activity of the Sorbit DES under various water contents at 50°C.

Water content (wt. %)	1.25	2.5	5	7.5	10
Viscosity ($Pa.s^{-1}$)	1.55	1.07	0.56	0.35	0.27
Water activity (A_w)	0.024	0.045	0.077	0.12	0.16

Statistical analysis of the different titers reached after 48 h in relation to enzyme concentration revealed significant differences when the amount of enzyme was at least doubled from the 20 g/L starting point (**Figure 8A**). Drastic differences are to be observed in **Figure 8B** as 0.5 M of vinyl laurate induces a two-fold increase in titer compared to 0.25 M and 0.75 M. This safely suggests that upon a range of concentration > 0.5 M, there was potentially an inhibition of the biocatalyst due to substrate saturation. Above this concentration and despite the statistical

analysis, comparisons are rendered ambiguous considering the lack of trend and should be interpreted with a certain distance.

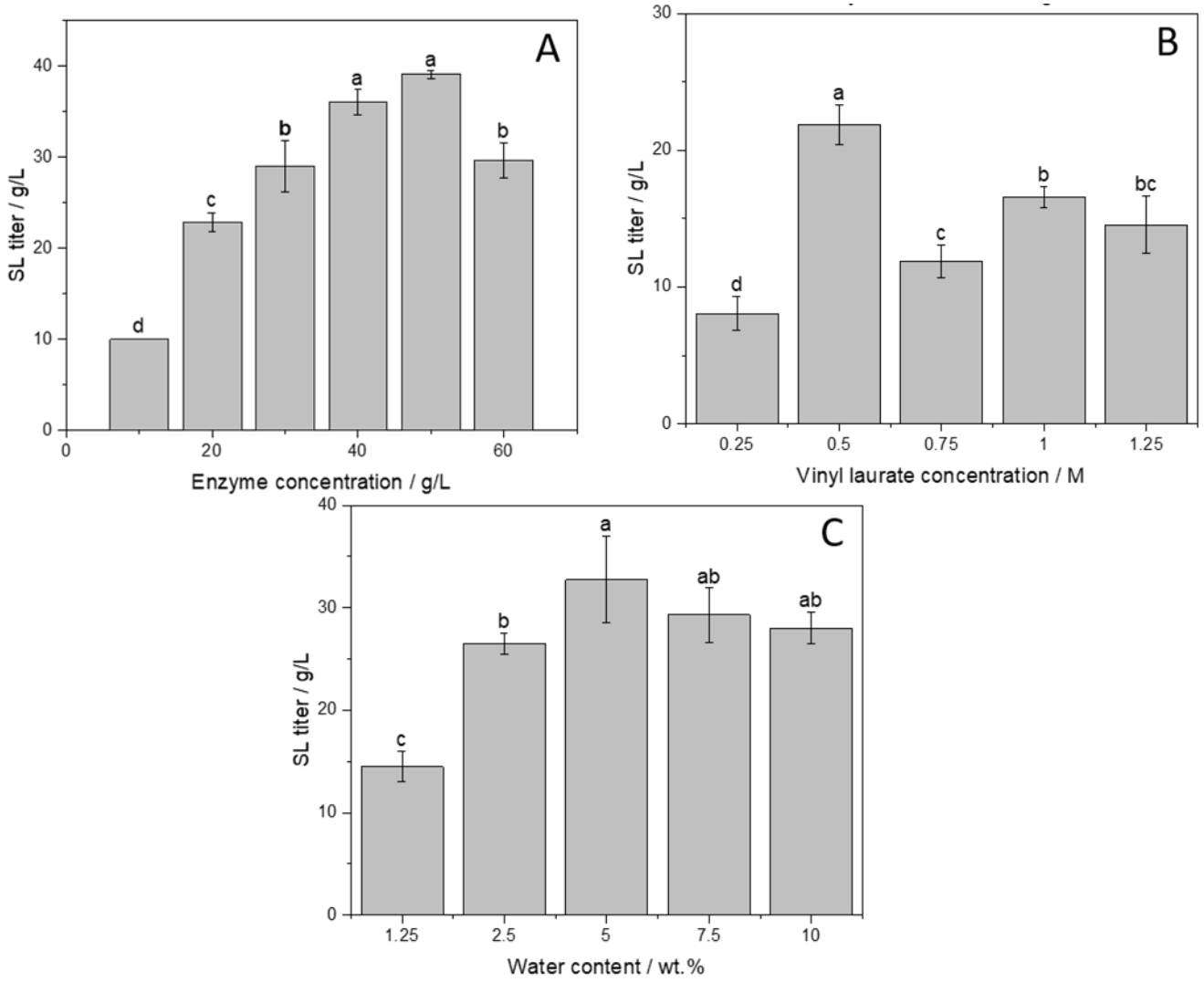


Figure 8. Novozym 435®-catalyzed transesterification of sorbitol and vinyl laurate in "2-in-1" Sorbit deep eutectic system: Effect of enzyme dosage (**A**); vinyl laurate concentration (**B**); and water content in the media (**C**) on the titer after 48 h. a, b, c and d show statistically significant differences, at a 0.05 significance level, of the mean values obtained from three independent experiments ran under each condition.

The results displayed in **Figure 8C** are rather more ambiguous as well, concerning the water content. Indeed, above 5 wt.% water, it seems that an optimal range of water content was reached. Overall, the results tend to indicate that enzyme and substrate concentrations were predominant factors compared to the water content, to some extent, as the Sorbit DES with a 2.5 wt.% water content ultimately limits the conversion most probably due its measured 2-fold higher viscosity (**Table 5**). It also seems that the Sorbit DES with 5 wt.% water provides the optimal A_w for the enzymatic activity (~0.08). Thus, a compromise has been found on the water content of the Sorbit DES, giving a good balance between viscosity and water activity for an optimal substrate conversion. In the following section we display the structural elucidation of the product and the impact of the optimized combined factors on our process.

2.3.5. Structural elucidation Using Spetroscopic and Spectrometric Methods

Here, we report one of the most extensive structural characterization of SL. ^{1}H and ^{13}C-Nuclear Magnetic Resonance (NMR) Spectroscopy and Mass Spectrometry (MS) were performed on the purified compound. Additional ^{1}H-^{1}H COSY and ^{1}H-^{1}H TOCSY, ^{1}H-^{13}C HSQC and ^{1}H-^{13}C HMBC (**Appendix: Figures A4, A5, A6 and A7**, respectively) combined with MS results, confirmed that only one acyl group (laurate adduct) was bound to a primary hydroxyl group of sorbitol. From the 1D- and 2D-NMR experiments, the chemical shifts and their assignments are fully detailed in **Table 6**.

Table 6. ^{1}H- and ^{13}C-NMR chemical shifts of sorbitol-6-O-laurate (SL) with their molecular assignments.

Molecular group	^{13}C Shift (ppm)	^{1}H Shift (ppm)	Multiplicity	Coupling (Hz)
Sorbitol				
$-C^{1}H_2-O-$*	66.66	4.35, 4.18	dd, dd	3.0, 11.5, 6.3
$-C^{2}H-$	70.69	3.91	m	-
$-C^{3}H-$	73.24	3.68	dd	5.28, 7.37
$-C^{4}H-$	70.27	3.93	t	4.84
$-C^{5}H-$	74.32	3.83	m	-
$OH-C^{6}H_2-$	64.25	3.71	m	-
Laurate				
$O=C^{1}-OH$*	174.46			
$-C^{2}H_2-$*	34.64	2.33	t	7.6
$-C^{3}H_2-$	25.46	1.61	m	-
$-C^{4}H_2-$	29.72	1.32	m	-
$-C^{5}H_2-$	30.12	1.28	m	-
$-C^{6}H_2-$	30.12	1.28	m	-
$-C^{7}H_2-$	30.12	1.28	m	-
$-C^{8}H_2-$	30.12	1.28	m	-
$-C^{9}H_2-$	30.12	1.28	m	-
$-C^{10}H_2-$	30.12	1.28	m	-
$-C^{11}H_2-$	23.22	1.29	m	-
$-C^{12}H_3$	14.35	0.88	t	7.00

Note. * Acylation site; d = doublet; t = triplet; m = multiplet.

To determine which primary carbon of the *meso* and pseudo-asymmetric polyol is bound to laurate, we measured a ^{1}H-^{13}C HSQC with high resolution in the direct dimension and no decoupling. Extraction of 1D slices along the respective carbon frequency allowed estimation of vicinal $^{1H-1H}J$ couplings.

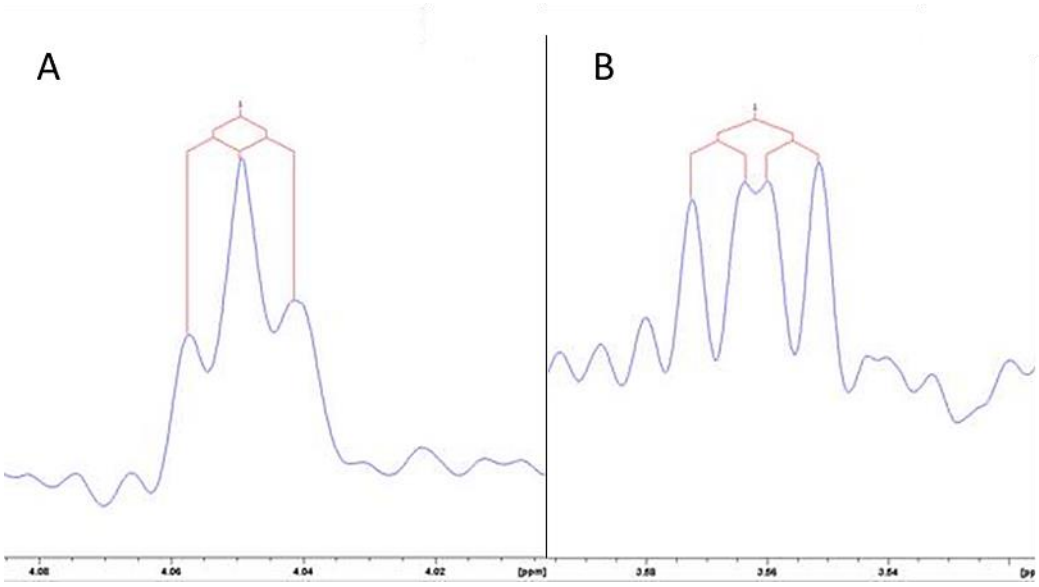


Figure 9. Extracted NMR signals of (A) C^4H and (B) C^3H of the sorbitol.

Figure 9 shows the signals that belong to C^4H and C^3H of the sorbitol. C^4H is a triplet with coupling constants of 4.8 Hz, indicating that both vicinal protons to this group are in *cis* position. C^3H on the other hand displayed a doublet of doublets with two different coupling constants of 5.3 Hz and 7.4 Hz, thus here the vicinal protons are different, best explained by one in *cis* and the other in *trans* position. This proves that the laurate adduct is connected *via* the ester function to the C^1H end of the polyol.

MS was performed with ElectroSpray Ionization (ESI) (**Appendix: Figure A8**). The spectrometric analysis of the synthesized SL is shown below in **Table 7**. In the latter, 5 clear adducts of SL have been observed and correlated to possible adducts.

Table 7. Adducts determined from the observed m/z obtained *via* ElectroSpray Ionization-Quadrupole (ESI-Q) experiment for the purified Sorbitol-6-O-Laurate (SL).

Observed *m*/z value	Corresponding adduct	Relative abundance (%)*
329.232	$[M_{SL}+H]^+-2H_2O$	7.27
347.243	$[M_{SL}+H]^+-H_2O$	100
365.253	$[M_{SL}+H]^+$	34.05
382.280	$[M_{SL}+NH_4]^+$	0.92
387.235	$[M_{SL}+Na]^+$	18.57

Note. *Calculated from the abundance of the ionic fragments, on the y-axis, that were produced by *in source* fragmentation.

2.3.6. Optimized Tube Scale Production and Scalability

Combining the optimized factors allowed a product concentration of up to 50 g/L of SL at the tube scale to be reached. Interestingly, to demonstrate scalability of the production in a batch STR, we ended up with a ~2-fold decrease of both titer and yield as shown in **Table 8**, suggesting that the reaction is sensitive to the effect of reactor. Presumably, the chosen parameters such as stirring speed and stirring did not match the performance of orbital shaking and resulted in lower performance. Albeit we demonstrated in the present work the scalability of our process. More investigation specific on this reactor system that would focus on these parameters is needed.

Table 8. Impact of optimized factors on SL titer after 48 h in Sorbit DES with 5 wt.% water, using Novozym 435® at tube and stirred-tank scales. Reaction conditions: 0.5 M vinyl laurate, 50°C, 50 g/L enzyme formulation.

Scale/Stirring	Titer (g/L)	Yield*** (%)	Specific reaction velocity*** (µmol/h/g)		
			4 h	24 h	48 h
Tube/Orbital Shaking	50 ± 3*	28 ± 2*	151 ± 13*	91 ± 4*	54 ± 5*
Stirred-Tank/ 3-bladed spiral propeller	25 ± 8**	14 ± 4**	134 ± 10**	40 ± 8**	29 ± 4**

Note. *n = 3, p-value < 0.05. ** n = 2, p-value < 0.1. *** Calculated from equations (1) and (2).

In **Figure 10**, we observe directly this blatant two-fold decrease in titer. However, it is interesting to notice an equivalent titer in the early stage of reaction being reached in the STR and the orbitally shaken tubes with comparable initial velocities. After 24 h, the difference in titer increases drastically while the specific productivity dropped in both reactors with a 1:2.5 ratio between the two systems. Finally, at 48 h, reaction velocities decreased again by roughly 2-fold conserving a similar ratio as previous. Thus, it is suspected that comparatively, enzyme was inhibited due to mass transfer limitations in the STR.

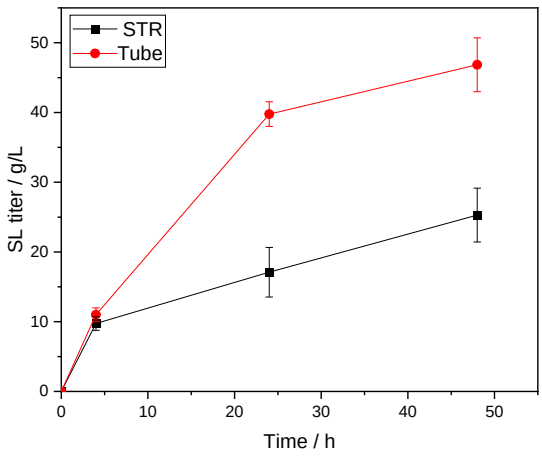


Figure 10. Time course of SL production (g/L): STR with 3-bladed spiral propeller versus tube in orbital shaking.

We propose thereafter a discussion making a parallel between our results and pre-existing ones in literature. Allowing us to give a broader aspect of the role of immobilized enzymes,

DESs, and reactor technologies in sustainable processes such as, but not only limited to, sugar ester synthesis.

2.4. Discussion

2.4.1 Immobilized Lipases and Media Tailoring for Sustainable Biocatalyzed Esterifications

To enhance the performance of the biocatalyst, immobilization of enzymes and more specifically lipases are of high industrial interest and it has become a requirement [140]. Indeed, it was shown in numerous reports that through *e.g.*, adsorption or covalent binding with a solid carrier, some acyltransferases, such as the lipase B from *Candida antarctica* (CaLB) could display improved activity, stability, and reusability [141–143]. This phenomenon can be understood *inter alia* as a gain of rigidity for the enzyme´s tertiary structure which limits protein unfolding and thus denaturation of the biocatalyst [144]. This is in practice true in the case of the Novozym 435® formulation that was recently qualified as the "perfect immobilized biocatalyst" in a review from Ortiz *et al*. [145]. In parallel, the native CaLB has been demonstrated to be among the most stable commercially available lipases [146,147]. By binding it to a macroporous acrylic polymer resin (Lewatit VP OC 16000), resulting in the now highly reported Novozym 435®, it increased drastically the performances of the biocatalyst [148].

In the current work, it might appear shocking that we use 50 g/L of enzyme to reach the same value in titer of product. At least on the tube scale, it is likely that in the case of Novozym 435® the costs associated with the immobilization might be fully compensated [149–159]. Indeed, the formulation can be recycled, using filtration, for sometimes up to 10 cycles as a report from Liu *et al*. demonstrates [160]. It is also important to remember that in such bead-adsorbed enzyme formulation, the non-catalytic ballast of the carrier represents a tremendous portion of the biocatalyst´s mass (90 to >99 wt.%) [148,161]. Thus, it is remarkable that in our work, the immobilized versions of CaLB such as Lipozyme 435® or Novozym 435®, rivalized with the buffered formulations as they should normally contain a higher enzyme concentration.

Interestingly, CLEA CA was in our case, comparable to the other CaLB formulations. The Cross-Linked Enzyme Aggregates (CLEAs) first introduced by Sheldon *et al*. [162], but also the Cross-Linked Enzyme Crystals (CLECs) [163] represent very interesting and more elegant alternatives to the petrol-based polymer carriers. CLEAs are a result of firsthand the precipitation and physical aggregation of the enzymes then secondhand the cross-linking of these aggregates with a cross linking agent which can be typically glutaraldehyde. This gives several advantages to CLEAs over their non-covalently immobilized analogues, such as the quasi-nonexistent leaching of enzyme even under reportedly harsh conditions [164]. Due to the covalent inter-molecular binding, CLEAs and CLECs afford complete removal of the carriers thus resulting into carrier-free immobilized enzymes. However, they also present limitations and challenges for their industrial scale production, notably for the control of the enzyme´s aggregation that can result in less active enzyme dimers [165], thus they are, to this day, rather rarely produced in bulk. On a side note, it is important to mention that standard acrylic-bead-immobilized enzymes were easier to handle, throughout the tube-scale synthesis and downstream processing than their CLEA analogues.

Herein, we reached an optimal titer after 48 hours of reaction which is, like other reports dealing with enzymatic production of sugar esters in DESs [119], but much shorter than

microbial fermentation to produce glycolipids. Indeed, as an example, to produce Mannosylerythritol Lipids (MELs), the average harvesting time is between 7 to 10 days for a titer ranging from 15 g/L to 165 g/L to obtain, in the case of the highest yields, complex mixtures of MELs [166–169]. Additionally, and as we demonstrated, fewer factors must be investigated when selectively producing tailor-made glycolipids using either free or immobilized enzymes. On the other hand, microorganisms, such as yeasts or fungi, exhibit much more complex behaviors that require acute control of the reaction conditions. In this specific case, and despite MELs being well established on the market, mixtures of products are often obtained, thus adding a degree of complexity to the DSP. Our process using a "2-in-1" DES as media is rather more straightforward comparatively, but more time and investigation are needed to overcome challenges to make it as relevant for industrial production. . In regard to DSP, it has been shown in several studies that DESs can be recycled as well [170–172]. In our case and for an efficient liquid-liquid extraction, the "2-in-1" DES was broken *via* aqueous solvation. It could be thus foreseeable, that choline chloride-rich wastes generated by such DES mediated process, could be re-valorized in feed additives [173] or as agrochemical active ingredients [174]. Unlike organic solvent, DESs do not have to undergo incineration thus their release in nature can be considered [175].

Analogously, a eutectic mixture using organic solvents (NaOH/DMSO/2M2B) was investigated, for the synthesis of SL using lauric acid, by Kim *et al*. reaching exceptionally high yields (97%) using ~500 g/L of enzyme [131]. Despite this remarkable achievement, we propose in contrast a method using a deep eutectic system made of ubiquitous, renewable, and inexpensive compounds such as choline chloride and sorbitol using 10 times less biocatalyst. Concomitantly, they also reported an adverse effect of highly viscous mixtures on the reaction which correlates to the decreased titer we obtained when the reaction´s water content was set to 2.5 wt.%. Overall, this might also explain our comparatively inferior conversion yields as it seems that deep eutectic systems present challenges in terms of mass transfer limitations, meaning that our substrate hardly moves to the enzyme´s active site. Zhao *et al*. reported a bisolvent system containing either ILs or DESs in combination with 2M2B for glucose-based fatty acid esters. Similar factors were investigated and gave results equivalent to ours such as an optimal enzyme content of 20 g/L of Novozym 435® at 60°C to reach 46 % conversion yield from the 0.3 M of vinyl ester used [53].

The ever-growing knowledge on enzyme immobilization and media-tailoring technologies have shown to be crucial tools to reach sustainability in biocatalysis. They are also of active interest for various industrial domains to develop greener and sustainable processes [126,176–179]. Regarding this aim and as performance is of keen interest for industrial application, reactor technology and scalability represent two important pillars as well.

2.4.2. Reactor Technology: Toward Scaling-Up Lipase-Catalyzed Reactions

The development of suitable reactors and technologies that support lipase-catalyzed reactions is a topical subject for both academia and industry. Some parameters inherent to reactors have been shown to be of high importance for the performance of the process. For example, the speed of stirring can drastically influence the initial velocity of reaction and the conversion yield. This was clearly demonstrated in a research article from Korupp *et al.* dealing with the enzymatic production of glycerol adipate using Novozym 435® [180]. In the latter, they observed highest conversion rates at 100 rpm. The type of stirrer did not significantly influence the reaction; however, it was observed that only a helicon ribbon stirrer gave a uniform

convection of both substrate and catalyst. Similar observation was made in our case using a 3-bladed spiral propeller instead of a 4-bladed flat turbine (unpublished data), suggesting that homogeneity of highly viscous mixtures can be reached optimally in an STR with stirrers that induce vertical convection due to a rather axial flow. Indeed, studies from Wiemann *et al.* and Ansorge-Schumacher *et al.* are suggesting that viscous mixtures requiring high energy input might have deleterious effect on the physical and mechanical properties of immobilized formulations [181,182]. To corroborate these affirmations, a study from Keng *et al.* used a Rushton turbine impeller providing this time a radial flow that was seemingly more adapted to the relatively lower viscosities of their *n*-hexane-based mixture [183]. In their report dealing with the Lipozyme RM IM-catalyzed palm esters synthesis, it was clearly concluded as well, that shear effect of high impeller speed on the enzyme particles caused adverse effect on reaction performances. Thus, potentially explaining our significant loss of titer (~2-fold decrease) and lower reaction velocity when scaling up from the tube to the STR (Table 5 and Figure 5). Altogether it seems that a compromise needs to be found on the stirrer type and its speed, to conserve the integrity of the biocatalyst. In this regard, more stable lipase formulations are commercialized to respond to this problem. A good example is the recently co-developed and industrially produced CalB immo Plus® from c-Lecta and Purolite companies (www.c-lecta.com/www.purolite.com). The highly hydrophobic carrier ECR1030M (DVB/acrylate copolymer) exhibits enhanced mechanical stability and offers controlled size of spherical beads.

As a matter of fact, we mostly demonstrate what conditions were the most intrinsically influential on the reaction, but further studies would be needed to optimize the process at an even bigger scale. We essentially brought in the current work, a proof of the scalability of our process and more generally the scalability of the, scarcely represented in the literature, DES-mediated biocatalysis [184]. Despite a lack of in-depth understanding on how mechanistically DESs can activate and stabilize lipases, the room for improvement of this topic toward industrial application is rather wide. However, we were able to demonstrate that preparative scale is reachable using a facile and straightforward method, that requires the minimum necessary equipment as shown in the flowsheet of **Figure 11**. We removed therein the need for continuous pH and gas composition assessment that are standardly used in microbial fermentation, among other measurements that require probes combined to their respective computer software.

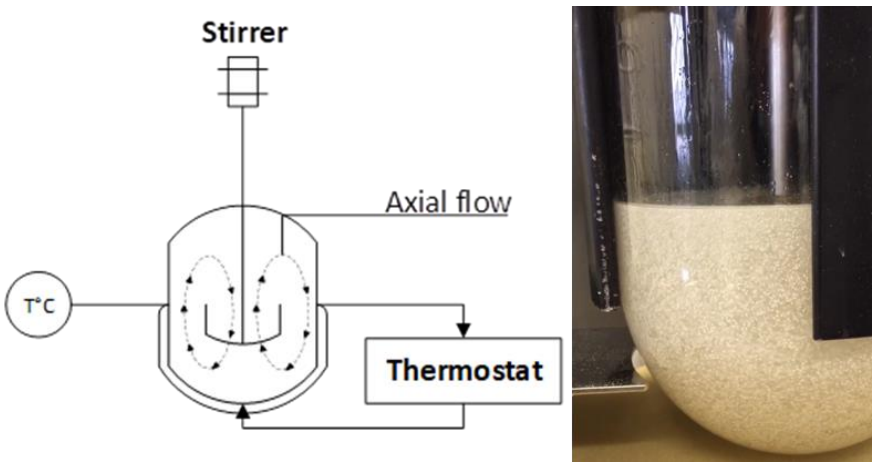


Figure 11. Flowsheet and picture illustrating the visibly homogenized lipase-catalyzed production of sugar alcohol monoesters using a stirred-tank reactor.

Although our discussion mainly revolved around the use of rather conventional reactors, such as STRs for batch heterogenous biocatalysis, innovative and commercially available alternatives have risen on the market. The Rotating Bed Reactors (RBRs), notably commercialized by the SpinChem company (www.spinchem.com), have proven to be promising alternatives to STRs and have shown good results when used for immobilized enzymatic reactions [185]. Furthermore, their cooperation with Purolite (www.purolite.com) gave light to immobilized lipase cartridges, theoretically forming a rotating packed bed reactor, that removes the need for filtration during the DSP and simplifies the recovery of the biocatalyst. Remarkably, packed bed reactors have also been combined with DESs for lipase-catalyzed esterifications. In a relatively recent study from Guajardo *et al*., they managed the transition from a batch, to a fed-batch and continuous process, enhancing simultaneously conversion yield and productivity thus seemingly resolving the encountered issue of mass transfer limitations and biocatalyst inhibition [186].

Recently as well, our research group published a proof of concept for the simultaneous extraction of lipids from yeast and the subsequent ester production in a "2-in-1" Sorbit DES, using microwave heating as an alternative to thermal heating [133]. This simplified and fast method is only an example of the vast possibilities that DESs, immobilized enzymes and innovative reactor systems can offer not only to the field of biocatalysis but also to biotechnology in a broader scope.

2.5. Conclusions

In this chapter, we presented the screening of 16 commercially available lipase formulations for the DES mediated and lipase-catalyzed production of glycolipid sorbitol-6-O-laurate. We determined the influence of several factors such as time of reaction, enzyme dosage, substrate concentration and water content when using Novozym 435® as biocatalyst owing to our newly HPLC-ELSD quantification method. We therein elucidated and identified, with complete spectroscopy and spectrometry analysis, the structure of the targeted compound. To finalize our report, we brought evidence of the possible scalability of such process and its importance for sustainable biocatalysis by highlighting analogous knowledge and facts from the literature. Overall, we show that despite many challenges and limitations, DESs demonstrate factually potential for mediating bioprocesses. Thus, it is foreseeable that more and more concrete applications will emerge concerning this specific topic.

3. Intensification of Immobilized Enzyme-Promoted Sugar Alcohol Ester Production in Heterogenous Unconventional Media Using Microwave Irradiation

This chapter is partly based on the publication draft:

Intensification of Immobilized Enzyme-Promoted Sugar Alcohol Ester Production in Heterogenous Unconventional Media Using Microwave Irradiation
André Delavault, Oleksandra Opochenska, Katrin Ochsenreither and Christoph Syldatk
To be submitted

Authors Contribution:

André Delavault: conceptualization, methodology, formal analysis, investigation, writing of the original draft, writing of the reviews, and editing

Oleksandra Opochenska: methodology, formal analysis, investigation, writing of the original draft, writing of the reviews, and editing

Katrin Ochsenreither: methodology, formal analysis, investigation, writing of the original draft, writing of the reviews, editing and supervision

Christoph Syldatk: supervision and funding acquisition

3.1. Introduction

Previous chapter demonstrated that enzymatic production of sorbitol laurate was rendered possible in a so called "2-in-1" Deep Eutectic Systems (DES), made notably of choline chloride in combination with sorbitol, implicated in the bioconversion as well. This principle was first reported by Siebenhaller *et al*. [127] and thereafter reviewed in an article from Pätzold *et al.* [126]. However, the performances of this specific proof of concept, using mainly glucose as acyl acceptor, were exceptionally poor (~4% yield). Recently, we managed to increase the conversion yield of this process by a 7-fold factor, compared to the original proof of concept by using a better acyl acceptor (sugar alcohol) and optimizing the synthesis factors. Scalability of the process was demonstrated as well but, in the meantime, mass transfer was *inter alia* highlighted as a potential limitation to the performance of the process during the scale-up to a batch stirred tank reactor. Indeed, not only an overall 2-fold loss of titer but also a drastic loss of specific reaction rate were observed after only 4 hours of reaction ending up in a rather slow reaction [187]. Thus, as a follow-up study, and in an aim of further intensification, we investigate herein the use of both microwave irradiation and organic solvents to increase the performance of glycolipid production. Comparisons between the different solvent systems and heating methods in overall yields and productivities are bringing thus nuance to the promotion of DES as green media for biocatalysis. Nonetheless, it is crucial to remember the intrinsic advantages of these low transition mixtures such as their formation, use, price and harmlessness [121,188–190]. Therefore, it is of interest to pursue efforts in making these innovative and sustainable media competitive to standard organic solvent in order to gain applicability and industrial interest [191].

Microwave heating is being under scrutiny as well, as it enhanced performances of numerous chemical reactions, extractions and biomass valorization processes [192]. However, it is rather scarcely represented in literature and it is still a niche topic for sugar esters production specifically [193]. It is a highly promising tool to intensify biocatalysed reactions as previous reports outline synergistic effect between the resulting dielectric heating and biocatalysts [194]. Indeed, it has proven to increase tremendously the reaction rates and overall yields of rather slow enzyme-catalyzed reactions [195–198]. We aimed at intensifying the lipase-catalyzed production of sorbitol laurate in different media following this principle. The synergism highlighted herein allowed superior results not only in our case but also in similar studies dealing with esterification and transesterification [199]. This phenomenon could be interpreted as a higher collision frequency between molecules, favorized intrinsically by localized superheating [200–202]. In the monomode reactor used in this study, the microwave radiations are typically guided through a rectangular wave guide towards the reaction container which is located at an interval from the magnetron that creates this standing wave (see **Figure 12**). As a result, an elevated microwave field density is created, causing extremely fast heating rates. Due to a uniform microwave field, no hot or cold spot is formed [193]. Similar study have shown that monomode reactors have been successful used for the synthesis of isoamyl myristate motivating thus our investigations [203].

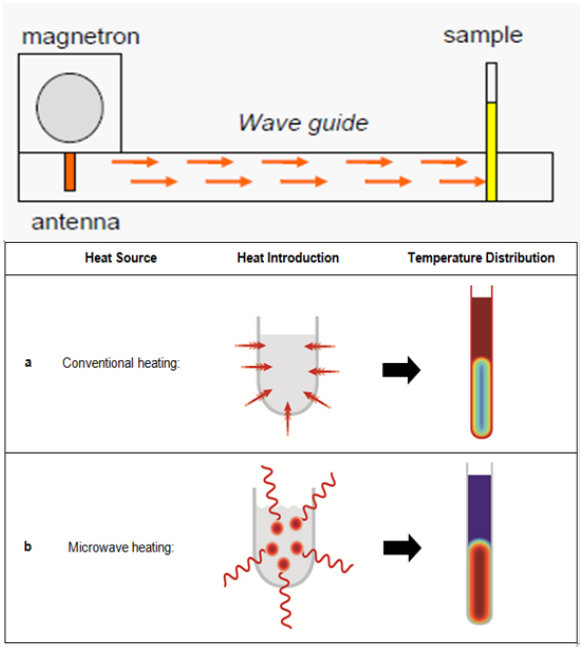


Figure 12. Principle of microwave radiation in monomode reactors and heat distribution of conventional heating vs microwave irradiation.

Conventional heating, in the other hand, transports the energy into the reaction *via* thermal conductivity of the reaction medium. Consequently, higher heating of the reactor than the reaction itself can be caused which is in opposition to microwave technology that allows an effective and internalized heating [204]. To complete our work the enzyme´s recycling as well as the efficiency of the liquid-liquid extraction using ethyl acetate was investigated.

3.2. Materials and Methods

3.2.1. Materials

Vinyl laurate was purchased from Tokyo Chemical Industry Co., Ltd. (TCI Europe, Belgium). Lipase formulation Novozym 435® was given by Novozymes (Denmark). All other chemicals and more notably solvents were purchased from either Carl Roth GbmH & Co. KG (Karlsruhe, Germany) or Sigma Aldrich Chemie GmbH (Taufkirchen, Germany) if not stated otherwise. In addition, all solvents were dried over $MgSO_4$ for 72h and stored with ~100 g/L of molecular sieves (3 Å) prior to use.

3.2.2. DES Preparation

The sorbitol and choline chloride based DES, dubbed "Sorbit DES", was prepared and validated according to the procedure described by Dai *et al*. and Hayyan *et al*. [137,138]. The water content was fixed at 5 wt.%

3.2.3. Solvent Systems Screening: SL Synthesis using Conventional Heating

In a 5 mL Eppendorf tube, were introduced subsequently:

For the organic solvent-mediated synthesis: 1.5 mL of warm Sorbit DES, vinyl laurate (195 µL, 170 mg, 0.75 mmol, 0.5 M) and 30 mg of enzyme formulation (20 g/L). The tubes containing the reaction mixture were agitated in a rotator and vortex mixer (program U2) from neoLab

(Heidelberg, Germany) at 90 rpm and 50°C. To get a triplicate for each measure, three tubes were collected for each time point at : 0.5h, 4h, 8h, 24h , 28h, 32h, 48h.

For the DES-mediated process: : 1.5 mL of warm Sorbit DES, vinyl laurate (195 µL, 170 mg, 0.75 mmol, 0.5 M) and 30 mg of enzyme formulation (20 g/L). The tubes containing the reaction mixture were agitated in a rotator and vortex mixer (program U2) from neoLab (Heidelberg, Germany) at 90 rpm and 50°C. To get a triplicate for each measure, three tubes were collected for each time point at : 0.5h, 4h, 8h, 24h , 28h, 32h, 48h.

All tubes were then processed for further analysis as described in subsection 3.2.8.

3.2.4. Influence of Biocatalyst, Sorbitol and Vinyl Laurate Concentrations in 2M2B

To examine the optimal concentration of lipase formulation, different concentration of Novozym 435® (10, 20, 30, 40, 50 and 60 g/L) were tested without varying any other reaction parameter.

To address the optimal substrates concentrations for the reaction, different vinyl laurate and sorbitol concentrations (0.25, 0.5, 0.75, 1 and 1.25 M) were tested independently from each other while all other reaction conditions were kept constant.

3.2.5. Intensification Using Microwave Irradiation and Influence of Temperature

In a 10 mL Microwave G6 tube, were introduced subsequently 3 mL of warm DES, vinyl laurate (390µL, 340mg, 1.5mmol, 0.5M) and 150 mg of Novozym 435® (50g/L). The tubes containing the reaction mixture were placed in the Monowave 400 (Anton Paar, Ostfildern, Germany) and incubated at 350 rpm and at temperatures of 50°C, 75°C and 100°C.

In a 10 mL Microwave G6 tube, were introduced subsequently 3 mL of 2M2B, vinyl laurate (585µL, 510mg, 2.25mmol, 0.75M), sorbitol (273.2mg, 1.5mmol, 0.5M) and 60 mg of Novozym 435® (20g/L). The tubes containing the reaction mixture were placed in the Monowave 400 (Anton Paar, Ostfildern, Germany) and incubated at 600 rpm and at temperatures of 50°C, 75°C and 100°C.

3.2.6. Enzyme Recycling

Reusability of Novozym 435® was tested in DES as well as in 2M2B. For both DES and 2M2B the reactions were performed under optimized conditions respectively. After 48h synthesis, the mixture was filtered using a Büchner funnel. The enzyme was washed with 4 x 20 mL distilled water, to dissolve the remaining sugar, and 4 x 20 mL of ethyl acetate to get rid of remaining DES. Then the enzyme was dried overnight in a desiccator provided with a drying agent. Afterward, the re-conditioned enzyme was reused for two more synthesis cycles with the same washing protocol in between. The residual activity in the following cycles was calculated as percentage of the conversion obtained in the first cycle.

3.2.7. Extraction Efficiency using Ethyl Acetate

To evaluate the extraction performances, 40 mg of sorbitol laurate was incubated in 1 mL of Sorbit DES for at least 1 h and afterward extracted and analyzed by HPLC-ELSD. The extraction efficiency was calculated as follows:

$$\text{Extraction efficiency [\%]} = \frac{\text{c SL}_{\text{extracted}}[\text{g/L}]}{\text{c SL}_{\text{added}}[\text{g/L}]} \times 100\% \quad (3)$$

c : concentration

3.2.8. Sample Preparation and HPLC-ELSD Quantification Method

Tubes containing organic solvents were, post-reaction, subsequently dry-evaporated on a SpeedVac centrifugal evaporator (H. Saur Laborbedarf, Reutlingen, Germany) and re-suspended in 1 mL of chloroform:methanol (75:25 v/v), the other tubes containing DES were prepared and analyzed *via* HPLC-ELSD as described by Delavault *et al* [187].

3.2.9. Data Treatment and Statistical Analysis

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

3.3. Results

In the following sections, we report the investigation of several solvent systems under conventional heating to produce SL alongside the investigation of the impact of several synthesis factors in 2M2B. Following the product formation over time also allowed optimization of reaction time, enzyme, and substrates concentrations. Then, we optimized further the process using microwave irradiation and compared the initial specific reaction velocities, at different temperatures, to those obtained with conventional heating. Finally, we studied the efficiency of our DSP over several cycles of liquid-liquid extraction using ethyl acetate and the effect of re-use of the biocatalyst in 2M2B and DES.

3.3.1. Comparing Solvent Systems for Lipase Catalyzed Production of SL

Figure 13 shows that the highest titers of SL were obtained in 2M2B using Lipase B from *Candida antarctica* (CA) formulation: Novozym 435®. Without optimization, the latter reaches almost ~100 g/L which corresponds to a relatively high specific productivity, at the tube scale, of 287 μmol/g/h. In comparison, the DES system shows lower performance but displays a quasi-inexistent volatility.

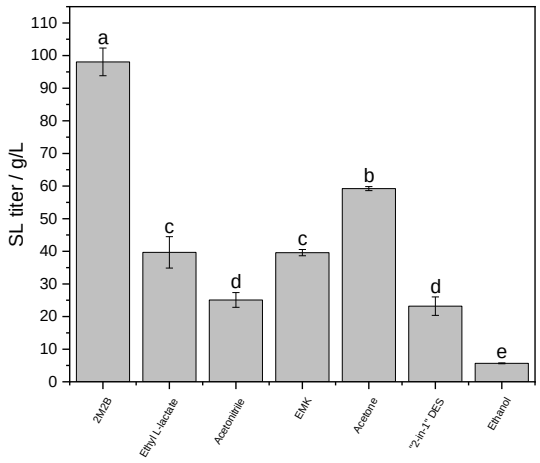


Figure 13. Comparison of SL titers calculated from 0.5 M vinyl laurate using Novozym 435®. A triplicate was done for each screened commercially available formulation after 48h at 50°C. a–e show statistically significant differences ($p < 0.05$).

Acetone allowed to reach an honorable 60 g/L titer which is significantly higher than other recent and green solvents such as ethyl-L-lactate, EthylMethylKetone (EMK) and sometimes forgotten as such, ethanol. Paradoxically, both alcohol solvents employed here showed drastic differences as 2M2B, a tertiary alcohol, is about 10 times as more effective as ethanol, a primary alcohol, to mediate the biocatalysis. The latter gave lowest titer in SL which could be attributed to a greater destabilization of the enzyme´s tertiary structure or a disruption of the enzyme´s essential water shell as ethanol is highly hydrophilic and water miscible. As 2M2B seemed a very promising option, we deemed necessary to look for the optimal reaction time, biocatalyst dosage and substrates molar ratio.

3.3.2. Factors Affecting the Synthesis in 2M2B and Optimized Process Performance with Conventional Heating

Herein, Novozym 435® was used as the biocatalyst for the reaction that was carried out in 2M2B. Factors impacting the reaction (i.e., time of reaction, substrates concentrations and enzyme dosage) have been investigated to find the optimal parameters for each condition. From **Figure 14**, it can be discerned that the optimal values were 8h, 20 g/L enzyme, 0.75 M vinyl laurate and 0.25 M of sorbitol.

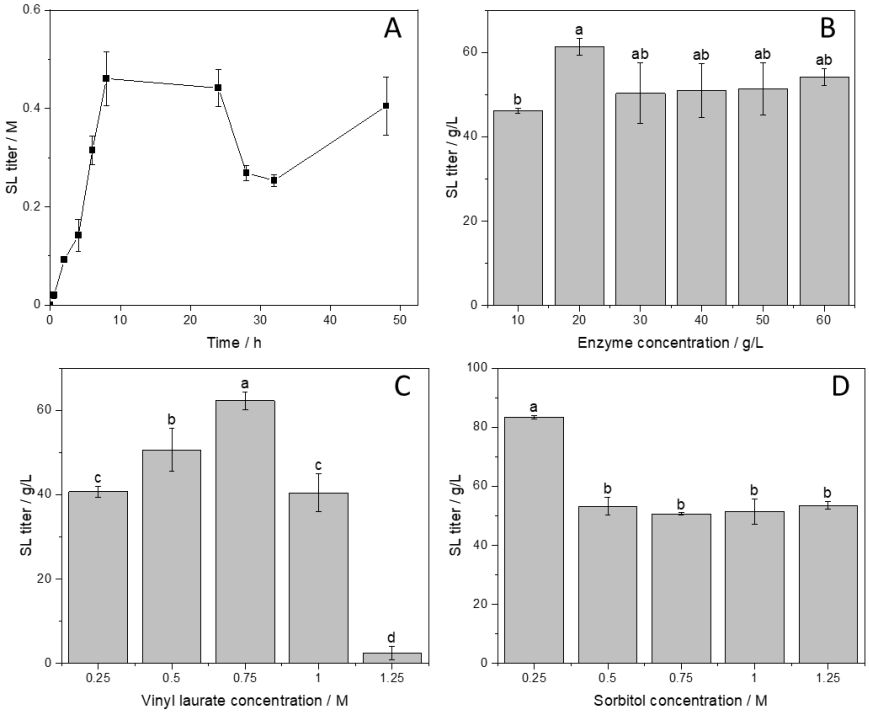


Figure 14. Novozym 435®-catalyzed transesterification of sorbitol and vinyl laurate in 2M2B: Production time course (**A**); Effect of enzyme dosage (**B**); vinyl laurate concentration (**C**); sorbitol concentration (**D**) on the titer after 8 h. a–d show statistically significant differences, at a 0.05 significance level, of the mean values obtained from three independent experiments ran under each condition.

Statistical analysis of the different titers reached after 8 h (**Figure 14A**) in relation to enzyme concentration revealed significant differences when the amount of enzyme was kept at the 20 g/L starting point (**Figure 14B**). Drastic differences can be observed as well, in **Figure 14C**, as 0.75 M of vinyl laurate induces a 1.5-fold increase in titer compared to 0.25 M and a 10-fold increase in comparison to 1.25 M. This safely suggests that upon a range of concentration >0.75 M, there was potentially an inhibition of the biocatalyst due to substrate saturation. The results displayed in **Figure 14D** are rather more ambiguous, and suggests that using 0.25 M sorbitol

induces higher titer of SL. Thus a 1:3 molar ratio of sorbitol to vinyl laurate seems overall more performant.

Combining the optimized factors allowed a product concentration of up to 168 g/L of SL at the tube scale to be reached in only 8 hours of reaction. Interestingly, when all conditions have been optimized for each solvent, this titer corresponds to a 3-fold increase in performance compared, regarding only overall yields, to acetone and DES as shown in **Table 9**, suggesting that the reaction is highly sensitive to the effect of the solvent. In consequence we end up with a dramatically superior specific productivity of 4.4 mmol/h/g.

Table 9. Impact of optimized factors on SL titer in 2M2B, acetone and DES after their respective optimal reaction times, using Novozym 435® at tube scale.

Solvent system	Optimal reaction time (h)	Productivity (g/L/h)**	Yield (%)***
2M2B	8	21 ± 2*	92 ± 4*
Acetone	48	1.25 ± 0.02*	33 ±5*
"2-in-1" Sorbit DES	48	1.1 ± 0.1*	28 ± 2*

*n = 3, p-value < 0.05. ** Based on optimal reaction time.*** Calculated from Equation (1).

Thus, the optimization of the factors affecting the synthesis and reaction time has boosted the performance of the process by 15-fold compared to the starting point using 2M2B that corresponds to a >90% conversion yield with conventional heating. Thereafter we investigated the use of innovative microwave technology to bring even further up the initial velocity of our reaction.

3.3.3. Intensification of the Process via Microwave Irradiation and Influence of Temperature

As outlines the **Figure 15** microwave technology allowed a tremendous increase in initial velocity of the reaction. However, this effect is only observed in 2M2B as seemingly the performance in DES suffers from a poor observed mixing. Thus, intensification using microwave technology is rather more suitable for reactions mediated by low viscosity solvents or adjuvants, unless a method to overcome poor mixing in DES is found.

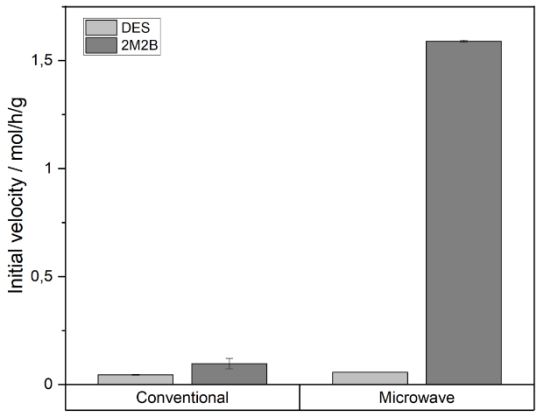


Figure 15. Initial reaction velocity in relation to the heating method and solvent system.

It also appears that in microwave, the enzyme meets its optimal activation energy at 75°C which allows an initial reaction velocity of 2.38 ± 0.26 mol/h/g (**Table 10**). As matter of fact, process intensification using microwave allowed a reaction 50 times faster in half the time needed to reach optimal speed using conventional heating with quasi-total conversion yields.

Performance in DES was slightly enhanced using higher temperature as it should logically decrease viscosity and enhance the reaction´s stirring but it is, in comparison, very minor to the observation made for 2M2B.

Table 10. Impact of temperature with microwave heating on initial (4 h) reaction velocity in 2M2B and DES.

Solvent system	Initial Reaction Velocity (mol/h/g)**		
	50 °C	75°C	100°C
2M2B	2.12 ± 0.56*	2.38 ± 0.26*	2.07 ± 0.37*
"2-in-1" Sorbit DES	0.09 ± 0.01*	0.05 ± 0.01*	0.04 ± 0.01*

*n = 3, p-value < 0.05. ** Calculated from Equation (2).

To conclude the results presented in this study, we also investigated the extraction efficiency using liquid-liquid extraction. Also, we highlighted the effect 2M2B and DES on the reusability of the biocatalyst.

3.3.4. Extraction Efficiency and Enyzme Reusability

To demonstrate the efficiency and the reliability of our extraction method, we used ethyl acetate, another green solvent alternative, over six extraction cycles to recover solubilized SL in the DES system **(Figure 16A**). Doing so, a two-phase system was formed that consisted of a DES-containing aqueous phase and an organic phase containing the studied glycolipid. After the first extraction, 70% of the product was recovered, however three additional cycles were needed to extract the quasi-totality of the remaining sorbitol monolaurate. Thus, an extraction yield >99% was reached after 4 steps. In comparison, the workup of the reaction in 2M2B only needed evaporation and re-solubilization

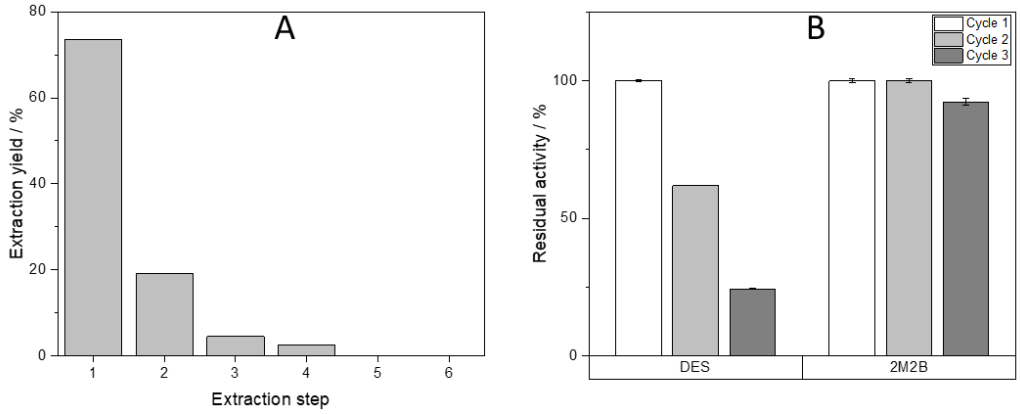


Figure 16. Extraction yield, over several extraction steps, of SL in diluted DES-ethyl acetate two phase system (**A**); residual activity of the enzyme in relation to the reaction cycle. Yield of reaction after 1st cycle was set to 100% (**B**).

Additionally, Novozym 435® was re-used for three cycles in both 2M2B and DES, each cycle with a total duration of 48h. Post reaction, filtration washing and desiccator drying of the enzyme allowed recovery of the bead-immobilized lipase. Consequently, we can compare the influence of both media on the biocatalyst using conventional heating (**Figure 16B**). After three cycles of effective use, 144 h in total, it seems that the biocatalyst retained most of its activity in 2M2B, which is greatly valuable for industrial application as it can drastically increase cost-effectiveness of the process. However, it seems that DES has a rather deleterious impact on the

enzyme´s stability as it loses about ¼ of its performance in each cycle. This could be either explained by a greater leaking of enzyme in DES or a greater destabilization of the enzyme structure such as protein unfolding or tertiary structure modification. Further studies, specifically in this system would be needed to clarify these hypotheses.

3.4. Discussion

3.4.1. Biocatalysis in Unconventional Media: A Gateway to Sustainability

As partly demonstrated in the current work, biocatalysis offers both economic and environmental advantages over chemocatalytic methods. Advantages that can be even further strengthen *via* immobilization and re-use strategy of the biocatalyst [145]. Indeed, enzymes are produced from inexpensive renewable resources and are biodegradable thus respecting intrinsically fundamental principles of green chemistry and sustainable developments [116]. In opposition to organo-metallic catalysts which demand metals like rhodium for asymmetric transformations in chemical synthesis which is one of the scarcest metals on earth [205]. Thus, in comparison, synthesis using instead enzymes is generally cheaper, facile and amenable.

Organic solvents have been used as reaction media for the synthesis of sugar alcohol esters in several studies. For example, sugar monoesters have been successfully produced from solid carbohydrates and vinylated fatty acids with high selectivity and high yield, in solvents such as acetonitrile, acetone, hexane and 2-methyl-2-butanol [206]. It was also reported, that 2M2B, as a solvent, is rather compatible with food and pharmaceutical applications, for the preparation of monoesters from different sugars and sugar alcohols [207].

One of the main issues in the enzymatic reaction is for solvent to show solubility for both substrates and retain the enzyme activity. Sorbitol solubility is very high in water and in many alcohol solvents due to formation of hydrogen bonds [130], but it is insoluble in most non-polar and aprotic solvents. Vinyl laurate is slightly soluble in water, 1 g/L at 20 °C, and well soluble in most alcohols. The log P parameter describes hydrophobicity of the solvent, which explains its tendency to partition between phases with different polarities. 2M2B is considered a polar solvent with log P value of 1.3, ethanol, acetone, acetonitrile and ethyl-L-lactate are also all polar with log P values of (-0.31), (-0.16), (-0.334) and (-0.18) respectively. EMK has the log P value of 3.9. It was shown that the active site of the *C. antarctica* lipase stays stable in non-polar solvents (log P > 4), while polar solvents (log P < 2) can interact with the active site and break the hydrogen bond between the amino acid residues important for lipase activity [208]. Solvents having non-polar nature (log P> 4) show improved effect as compared to the polar solvents (log P< 2) on the performance of enzyme. As in our case, polar solvents such as Sorbit DES and 2M2B displayed fitting characteristics for the intensification using microwaves, further investigation was focused on them as nonpolar solvents have fewer interactions with dielectric heating.

The results presented in his work show very high conversion yield and initial velocity even for the unoptimized transesterification reaction of sorbitol and vinyl laurate in 2M2B. Both substrates are soluble in the solvent, indicating comparatively lesser mass transfer limitations, which can be attributed to its lower viscosity (3.79 mPa·s at 25°C). The high conversion yield was also reached in shorter time (8 h) compared to the reaction in DES. This also correlates to a report by Marathe *et al*. in which reaction equilibrium was reached in 4 h [209]. However, in our study, prolonged incubation time seem to lead to product degradation as titer decreased

after the 8h benchmark. Despite all the aforementioned advantages of 2M2B over DES, it is important to consider its physical properties, such as flammability and irritancy. Considering the biocompatibility as well as the sustainability of natural DESs, they represent a compelling alternative reaction media for production of biomolecules making them worth further research aiming at industrialized applications [210,211].

3.4.2. Intensification of Biocatalysed Processes Using Microwave Technology

Microwave technology is gaining popularity in the chemical industry due to its ability to allow high rates of conversion and space-time yields. Moreover, when compared to conventional heating, enzymes like *Candida antarctica* lipase B show improved stability under microwave irradiation [212,213]. When using conventional heating methods, transesterification reaction requires comparatively much longer reaction time, often even up to several hours, and higher temperature because of the lower thermal transmission efficiency. However, microwave coupled to ultrasound irradiation have potential to enhance transesterification and complete the reaction within shorter time span [214]. Compared to conventional heating method, microwave irradiation is efficient, fast, convenient and energy-saving [215].

To successfully apply microwave technology to a chemical process, solvents with strong microwave absorption properties are necessary. The two main solvent properties accountable for the microwave dielectric heating response are the intrinsic polarity and the ionic behaviour. Additional desirable physico-chemical properties are high thermal stability and low vapor pressure of the media. Microwave-assisted processes using DESs as solvent media can offer several advantages such as low energy consumption, fast processing times and high dissolving properties [216]. Thus, in our case, both Sorbit DES and 2M2B were suitable for the application of microwave irradiation as they respond to these requirements.

DESs also have other advantageous properties in relation with microwave requirements, such as high thermal and chemical stabilities, low vapor pressure, low flammability, and high solvation capacity [217]. In addition, they have many advantages over ILs and organic solvents such as low price, high biodegradability and low toxicity.

As mentioned earlier, there is a direct absorption of energy by the functional groups having ionic conductivity or dipole rotational effect under microwave irradiation. This absorption increases the reactivity of the chemical functions with surrounding reactants compared to convective incubation at the same temperature [218]. Also, as there is always trace of residual water in immobilized enzyme beads, which gets rapidly heated due to thermal effects (dipolar and charge polarization) and specific effects (purely non-thermal), it ends up in an improved enzyme activity [219].

As we presented in the result section, initial velocity of transesterification reaction in DES is 20% higher under microwave irradiation compared to conventional heating. This increase is not dramatic; however, it might be due to poor mixing properties of the magnetic stirrer in the microwave in comparison to the orbital shaking. Seeing, as the initial reaction velocity in 2M2B increased 16-fold under microwave irradiation in comparison to conventional heating, the issue seems to revolve around mass transfer limitations rather than enzyme degradation. On the contrary as report from Naushad *et al*. shows, DESs have the ability to keep the enzyme from irreversible aggregation at high temperatures and thus increase its thermal stability [220]. Compared to polar organic solvents, which potentially can disrupt the 3D structure of the

enzyme [208], the hydrogen bonding in DESs helps to preserve it at high temperatures. Furthermore, by absorbing the heat from the reaction medium, it helps maintaining the integrity of the native conformation of the enzyme and preventing the collapse of the enzyme tertiary structure a higher temperature [221]. Thus, it is assumed that ensuring proper homogenization of reaction media would enhance the conversion rate and concentration of produced glycolipid.

When we raised the reaction temperature up to 75°C, the initial velocity of the SL synthesis in 2M2B increased ~1.5-fold, which indicates elevated activity and expanded thermal stability of the enzyme. However, further increase of the temperature up to 100°C resulted in lower titer. As the reported optimal temperature range of Novozym 435 lies between 30°C and 80°C, the decrease in titer can be explained with denaturation of the biocatalyst.

In the case of transesterification reaction in DES at higher temperatures, the initial velocity decreased. It is believed that the trend of deactivation determined in this work might be due to the destabilization of reaction intermediate complexes such as the enzyme-substrate one [216]. However, weak motion in the mixtures could potentially lead to formation of uneven heating spots and temperature gradients, which results in enzyme denaturation. Heat provided by the microwave irradiation induced potential disruption and breakdown of weak ionic and hydrogen bonds. Report provided by Elgharbawy *et al*. suggests, that the presence of natural sugar-based DESs is preventing the enzyme solution from rapid dehydration at elevated temperatures [222]. Moreover, the natural sugar-based DESs also create a cohesive coating for the protein molecules that maintain the heat associated with the rapidly hydrating sample. It is possible that addition of a polymeric coating to enzyme formulations enhances enzyme stability by preventing/reducing protein-surface interactions. This also has been detected for protein formulations when adding low molecular weight surfactants, e.g., polyoxyethylene sorbitan esters [223].

Liu *et al* reported, that Novozym 435® can be recycled, using filtration [160], which was also demonstrated up to three cycles in this work. The catalytic activity of the enzyme in the 2M2B remained high with rising number of cycles, however this was not the case with the reaction in the DES. The residual activity achieved during the third cycle indicates a ~75% loss of performance from the biocatalyst. This have been caused potential by insufficient enzyme washing and removal of residual DES inside the porous carrier. If parts of unbroken DES or sugar remained on the enzyme carrier, this may strongly hinder its catalytic activity as it could obstruct the access to the activity pocket for substrates.

Still in an aim towards industrialization, Morschhäuser *et al*. demonstrated the first general purpose industrial scale continuous flow microwave reactor which allowed the safe and highly energy efficient processing of organic reaction or solvent-free mixtures under high temperature/high pressure conditions. The authors noted that reactor can also be used at moderate temperatures that are relevant for the synthesis of pharmaceutical intermediates [224] which could transferable to biocatalysis.

3.5. Conclusions

The results of lipase-catalyzed synthesis of SL in DES were compared to the one carried out in organic solvents which were more profitable in terms of production yields and reaction time. Thus, not only limitations were highlighted but also research axis for a further intensification

of enzymatic processing in DES were highlighted. Both solvent systems were put to the test using this time, microwave irradiation alongside temperature variation to investigate the influence of heating method on the glycolipid production. The results presented in this chapter only apply to 10 mL batch but scale up of this process could potentially open new horizons for industrial biocatalysis.

Overall, use of DESs as a reaction media comes with many challenges and limitations. This work demonstrates that performing lipase-catalyzed glycolipid synthesis in organic solvent can be more profitable not only due to higher yields but also to shorter reaction time and simplified downstream processing. DESs demonstrate nonetheless, through an ever-expanding literature, their genuine potential for mediating sustainable bioprocesses and reduce waste management strategies. Thus, further investigation and development are necessary to overcome hindrances intrinsic to DESs.

.

4. Microwave-Assisted One Pot Lipid Extraction and Glycolipid Production from Oleaginous Yeast *Saitozyma podzolica* in Sugar Alcohol-Based Media

This chapter is partly based on the publication:

Microwave-Assisted One-Pot Lipid Extraction and Glycolipid Production from Oleaginous Yeast *Saitozyma podzolica* in Sugar Alcohol-Based Media
André Delavault, Katarina Ochs, Olga Gorte, Erwann Durand, Christoph Syldatk and Katrin Ochsenreither
Molecules, 2021, 26 (2), 470.
doi: 10.3390/molecules26020470
Published: 18.01.21

Authors Contribution:

André Delavault: conceptualization, methodology, formal analysis, investigation, writing of the original draft, writing of the reviews, and editing

Katarina Ochs: methodology and investigation

Olga Gorte: methodology, investigation, writing of the original draft, writing of the reviews, and editing

Christoph Syldatk: supervision and funding acquisition

Erwann Durand: methodology, investigation, writing of the original draft, writing of the reviews, and editing

Katrin Ochsenreither: methodology, formal analysis, investigation, writing of the original draft, writing of the reviews, editing and supervision

4.1. Introduction

Surfactants are chemicals that are in an ever growing and worldwide demand in various fields of application like pharmaceutics, cosmetics, cleaning agents, or even the petroleum industry [114]. It is also reasonable to think that the recent Covid-19 crisis might boost this demand because of the use of such compounds in washing and more generally in hygiene products [225]. Such context makes it crucial to design processes in agreement with green chemistry principles that aim to synthetize amphiphilic molecules that are not only bio-sourced, but also biodegradable, using innovative and low energy input methods [226]. Glycolipids are in this regard an interesting class of surfactants since they are obtained *via* chemical or bio-chemical combination of a sugar, or sugar alcohol moiety, and a hydrophobic tail which can be a fatty acid, or a fatty alcohol. They are viable allies for greener surfactant production as the chemical functions that link the polar head and apolar tail are inherently biodegradable [227,228]. Indeed, in the chemical function bonding, those two components can be an ester when a lipase is supplied with a fatty acid playing the substrate role. Certain lipases, or acyltransferases, present the advantage to be useable in mild conditions and allow sugar ester synthesis when placed in water free conditions. We present here a potentially low energy input and simple process combined with a fast microwave-assisted preparation of low water content Deep Eutectic Systems (DESs) that uses constant and accurate control of the temperature coupled with an efficient diffusion of heat. Microwave-assisted chemistry is an environmentally friendly approach that can overcome limitations such as uneven heating, poor mixing, thermal stability of reagents, and high viscosity [216]. Such technology can be considered as a powerful, versatile, and flexible tool for chemical reactions, biomass treatment, and biocatalysis, which match with deep eutectic systems [229].

DESs were first proposed by Abbott *et al*. as alternative solvents to ionic liquids (ILs) [230]. At a certain molar ratio of the components, there is a significant lowering of the melting point, which results in the formation of a liquid at room temperature [61]. Our one-pot process used an acidified DES made of choline chloride and sugar alcohols, such as xylitol and sorbitol, to extract lipids from oleaginous biomass (*S. podzolica (Saitozyma podzolica)* DSM 27192). Subsequently, sugar alcohol esters were produced through lipase-catalyzed reaction. Palmitate, stearate, oleate, and linoleate monoesters were identified by spectrometric and spectroscopic methods. The overall strategy of our method is shown in the flowchart of **Figure 17**. A similar process was demonstrated by Siebenhaller *et al*., in which sugar esters were synthesized using a DES based on renewables. However, such integrated production required many steps like any other "Stop-and-go" process [135]. Parallelly, lipid extraction of oleaginous yeast cells is mostly carried out by a combination of cell disruption and extraction processes [231]. Mechanical, physical, chemical, and enzymatic methods have been used for cell disruption [232]. The most common methods for extraction are solvent system-based using chloroform-methanol [233] or ethanol-hexane [234]. It appeared therefore logical to look alternatively for greener and more innovative systems.

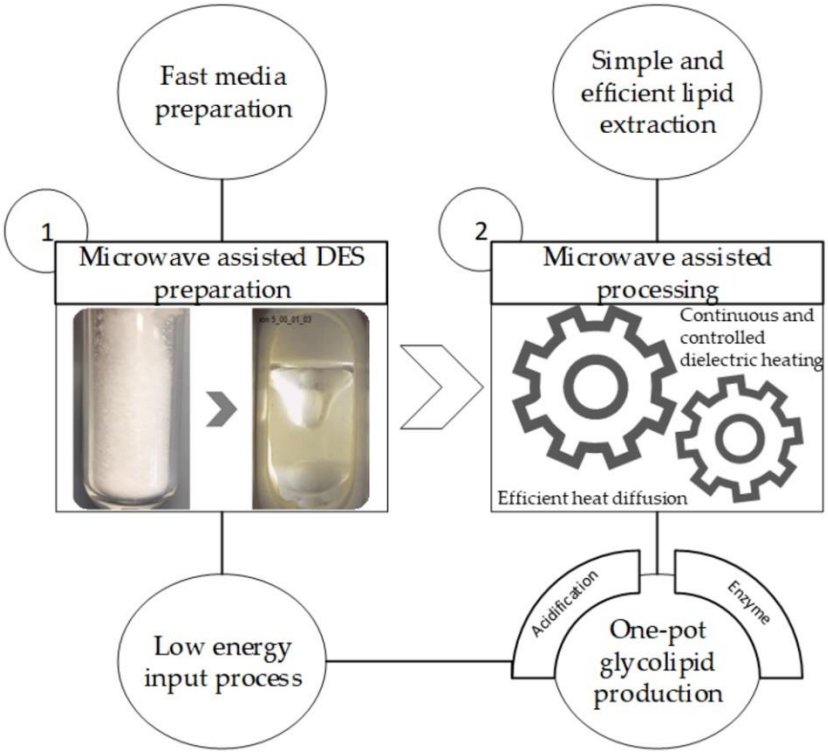


Figure 17. Flowchart of this study. Freeze-dried oleaginous biomass from S. podzolica was used in a one-pot microwave-assisted process that extracted fatty acids and subsequently produced a purifiable quantity of glycolipids when lipase and acidified Deep Eutectic System (DES) were jointly used.

Previous work from Gorte *et al*. demonstrated, in this aim, the use of several methods for the pre-treatment of the biomass produced by *S. podzolica*, including multi-step use of mechanical techniques such as ball milling, ultra-sonication, and high–pressure homogenization [231]. In the present work, we shortened the biomass treatment process through a single step approach, after freeze-drying the biomass, removing therefore the cell disruption step. We introduced the media, the biomass, and the enzyme, all together in one reactor vessel assisted by microwave irradiation. Thus, we reduced the number of steps, and allowed both the lipid extraction and the production of sugar alcohol esters without stopping the reaction or changing the apparatus. The extraction efficiency was compared to a standard Folch extraction, and the amounts, along with the profile of extracted lipids, were investigated.

It was originally expected that enzymatic or auto-catalytic acylation reaction between sugar alcohols composing the DESs and fatty acids of the biomass would have happened without prior modification of the components. Acidified DES (AcDES) showed, compared to a standard DES, an improved lipid extraction alongside a clear formation of several components that were identified as being primarily mono-acylated sugar esters. The following results show the enhancement of DESs made of sugar alcohols and choline chloride for the lipid extraction from *S. podzolica* biomass and subsequent sugar alcohol ester formation when supplementing acid and Novozym 435® as biocatalyst in the system. The presented process can be carried under classical heating conditions; however, the microwave reactor provides accelerated media preparation alongside controlled heating and cooling conditions that end up in an eased handling of the viscous mixtures.

4.2. Materials and Methods

4.2.1. Materials

Lipase formulation Novozym 435® was given by Novozymes (Denmark). Lipid-containing yeast biomass was produced by fermentation as described below. All other chemicals were purchased either from Car Roth GmbH & Co. KG (Karlsruhe, Germany) or Sigma Aldrich Chemie GmbH (Taufkirchen, Germany) if not stated otherwise.

4.2.2. Microorganisms

The oleaginous basidiomycete used in this study was screened and deposited at the DSMZ culture collection (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Brunswick, Germany) as *Cryptococcus podzolicus* DSM 27,192 by Schulze *et al.* [235]. After genome sequencing and annotation, the latter was phylogenetically reclassified to *Saitozyma podzolica* DSM 27192 by Aliyu *et al.* [236].

4.2.3. DES Preparation with Microwave Dielectric Heating and Conventional Convective Heating

Formation of DESs was validated as reported by Dai *et al.* [137] and Hayyan *et al.* [138]. In a G30 Anton-Paar microwave vessel, DESs compounds were introduced according to the quantities in **Table 11**. The resulting mixture was manually homogenized with a spatula, then placed into the microwave synthesizer (Monowave 400, Anton-Paar, Ostfildern, Germany) where it was heated to 95 °C with slow heating mode for DESs made of actual sugars and rapid heating mode for the others (850 W, stirrer speed 600 rpm) with IR thermometer.

In a 50 mL Schott flask, DESs components were introduced according to **Table 11**. The resulting mixture was manually homogenized with a spatula, then placed into a Thermotron incubator (Infors HT, Bottmingen, Switzerland) where it was heated to 95 °C at 300 rpm orbital shaking.

When clear homogenous phases were obtained, shaking and heating were stopped as the time was either given by the microwave software or simply noted from incubator´s timer.

4.2.4. Production of Single Cell Oil in Bioreactors

S. podzolica was cultivated in a 2.5 L Minifors bioreactor (Infors HT, Bottmingen, Switzerland) as described by Schulze *et al.* [235].

4.2.5. Microwave Processing of the Oleaginous Biomass

In an Anton-Paar G30 microwave vessel, were simultaneously introduced 10 mL of 5% 2M HCl (0.1 M) or 5% deionized water sugar alcohol-based DES with or without 200 mg of Novozym 435® (Novozymes, Bagsværd, Denmark) and 400 mg of lyophilized yeast powder. The reaction was then heated at 60 °C as fast as possible for 72 h with the Anton-Paar Ruby Thermometer (Anton-Paar, Ostfildern, Germany) (850 W, stirrer speed 600 rpm until temperature was reached, then 300 rpm). As a control, fatty acid methyl esters (FAMEs) were used as lipid source for the glycolipid production as described by Siebenhaller *et al*. [135].

4.2.6. DownStreamProcessing (DSP) and Flash Chromatography Purification of the Reaction´s crude

Once the reaction finished, the resulting mixture was dissolved in 10 mL of deionized water. The mixture was filtered with a Buchner funnel and the solid was washed with 3 × 5 mL water and with 3 × 5 mL of ethyl acetate. Then, 10 mL of brine was added, the aqueous phase was extracted with 6 × 25 mL of ethyl acetate in a separatory funnel, the organic phases were then gathered and dried over $MgSO_4$ for 15 min. The solid chemical dryer was filtered-off with filter paper and the solvent was evaporated with a rotary evaporator to obtain a brownish paste.

The crude paste obtained was re-dissolved in chloroform to be adsorbed over 4 g of silica (Kieselgel 60) for flash chromatography purification using the solid loading method. To purify this crude, a Reveleris PREP purification system equipped with a 4 g Flash Pure spherical column was used. Elution solvents were chloroform and methanol with a gradient such as: 2nd solvent percentage started at 0% for 1.5 min, 7% for 9.5 min, 15% for 3 min, and finally 100% for 3 min. The first fraction was fatty acids collected at 3 min. The second fraction was glycolipids collected at 4.5 min. The collected fractions were gathered and dry evaporated on a rotary evaporator for further analysis.

The total lipids per cell dry weight (CDW) were then calculated using the following formula:

$$\%\text{Lipids per Weight (CDW)}[\%] = \frac{\text{Lipid extracted [mg]}}{\text{Weight CDW [mg]}} \times 100\%. \qquad (4)$$

4.2.7. Folch Extraction and Direct Acidic Transestrification of the Biomass

To establish a base of comparison between methods for the extraction of lipids from oleaginous biomass, a Folch extraction was performed as follows. First, 400 mg of freeze-dried yeast biomass was homogenized by 45 sec vortexing with 8 mL of chloroform/methanol (2/1) in a 15 mL Falcon tube. After dispersion, the whole mixture was agitated during 20 min in an orbital shaker at room temperature. The homogenate was filtrated with a funnel provided with filter paper. After centrifugation and siphoning of the upper phase, the lower chloroform phase containing lipids was dry evaporated under vacuum in a rotary evaporator to be analyzed and profiled using GC analysis.

As a reference for the lipid content comparison, direct acidic transesterification of the biomass was done, as described by Gorte *et al*. [231].

4.2.8. Acidic Transesterification to Fatty Acid Methyl Esters (FAMEs) of the Lipid Fraction

The dried lipid fraction purified by the flash chromatography was dissolved in 1.5 mL of hexane. The solution was transferred to a glass tube and 0.5 mL of the internal standard (2 mg/mL heptadecanoic acid) and 2.0 mL of 15% H_2SO_4 in methanol were added. In addition, a blank was prepared in a glass tube consisting of 1.5 mL hexane, 0.5 mL internal standard, and 2.0 mL 15% s H_2SO_4 in methanol. The glass tubes were then incubated for 2 h at 100 °C and 1000 rpm in a thermal shaker. In between, the samples were homogenized by hand at intervals of about 30 min. After incubation, the glass tubes were cooled on ice for at least 5 min. Then, 1 mL of MilliQ water was added to the glass tubes and centrifuged at 2500 rpm for 5 min. Finally, 1 mL from the upper phase was transferred to a GC vial.

4.2.9. GC Analysis of Fatty Acid Methyl Esters (FAMEs)

The quantitative and qualitative analyses of the transesterified FAMEs were performed gas-chromatographically using the 6890 N Network GC-System (Agilent Technologies Deutschland GmbH, Waldbronn, Germany). The device was coupled with a DB-Wax column (122–7032; l: 30 m d: 0.25 mm, Agilent Technologies Deutschland GmbH, Böblingen, Germany) and the detection was performed with an FID under 1.083 bar working pressure. Then, 1 mL of sample was injected at the initial temperature of 40 °C. The separation of the FAMEs was achieved by a temperature gradient from 40 to 250 °C with a rate of 8 °C/min and was kept for 10 min at 250 °C. To identify and quantify the FAMEs, the RM3 FAME Mix standard (07256-1AMP; Sigma Aldrich, Taufkirchen, Germany) was used. Chromatogram and integration of the main signals are given in **Appendix: Figure A9**.

The yield of FAMEs and the FAMEs per CDW [%] were then calculated using the following formulae:

$$\text{FAMEs [mg]} = \text{extracted whole lipids [mg]} \times \text{FAMEs per whole lipid [\%]}, \quad (5)$$

$$\text{FAMEs per CDW [\%]} = \frac{\text{FAMEs [mg]}}{\text{CDW [mg]}} \times 100\%. \quad (6)$$

4.2.10. Thin Layer Chromatography (TLC) Analysis of Reaction Mixtures

After the DSP of the resulting mixtures, 10 mg of each reaction´s crude was dissolved in 1 mL of chloroform/methanol (75/25) and further used for TLC analysis as follows. First, 10 µL of the previously extracted organic phase was spotted onto a silica plate (Alugram SIL G, 60 Å, Macherey-Nagel GmbH & Co. KG, Düren, Germany). The eluent consisted of chloroform: methanol: acetic acid (65:15:2 v/v) [119]. After elution, TLC plate was dived into anisaldehyde: sulfuric acid: acetic acid (0.5:1:100 v/v) dying solution and subsequently revealed with a heat gun.

4.2.11. Spectroscopic and Spectrometric Methods for Structural Elucidation of Glycolipids

The nuclear magnetic resonance (NMR) spectroscopy spectra were measured in dichloromethane-*d*$_2$ (CD_2Cl_2 purchased from Eurisotop, Saarbrücken, Germany) on a Avance 400 NMR instrument (Bruker Biospin GmbH, Rheinstetten, Germany), with 400 MHz for ^1H. The chemical shifts are expressed in δ (ppm) versus tetramethylsilane (TMS) = 0. The mass spectrometry (MS) for mass identifications was performed with electrospray ionization (ESI) on a quadrupole Q Exactive Plus (ThermoFisher Scientific GmbH, Kandel, Germany) and recorded in positive mode. Raw data from MS and NMR was treated using MestReNova Suite 2020 [version 14.2.0].

4.2.12. Statistical Analysis

OriginPro software 9.7 [version 2020] (OriginLab Corporation, Northampton, MA, USA) was used for raw data treatment and statistical analysis. One-way ANOVA followed by post hoc test Tukey were performed using *p*-value <0.05.

4.3. Results

The present work investigates and compares the preparation of several DESs (**Table 11**) obtained with a microwave dielectric heating versus a conventional convective thermal heating method. The extraction efficiency was investigated by the calculation of Fatty Acid Methyl Esters (FAMEs) and whole lipid per Cell Dry Weight (whole lipid/CDW), alongside the profiling of the lipids. Finally, structures of produced mono-acylated sugar alcohol esters were assessed using spectrometric and spectroscopic methods.

Table 11. Composition of common and sugar alcohol-based DESs studied.

DES Number/Name	HBD	mR [mol]	m_{HBD} [g]	HBA	mR [mol]	m_{HBA} [g]	mR [mol]	m_{water} [g]	wt% Water
1	Arabinose	1	6.45	ChCl	1	6.00	0.8	0.62	5
2	Glucose	1	5.00	ChCl	2	9.69	2.50	1.25	9
3	Glycerol	2	7.86	Betaine	1	5.00	1.00	0.77	6
4	Urea	2	4.30	ChCl	1	5.00	-	-	-
5	Glycerol	2	6.60	ChCl	1	5.00	-	-	-
6	1,2-Propanediol	1	2.54	ChCl	1	4.65	1.00	0.60	8
7	1,4-Butanediol	4	6.15	Betaine	1	2.00	1.00	0.31	4
8	Saccharose	1	5.00	ChCl	4	8.16	4.00	1.05	8
Xylit	Xylitol	1	5.45	ChCl	1	5.00	0.8	0.52	5
Sorbit	Sorbitol	1	6.53	ChCl	1	5.00	0.9	0.58	5

Note. Final volume of DES results in 10 ± 1 mL, HBA: hydrogen bond acceptor, HBD: hydrogen bond donor, mR: mole ratio.

4.3.1. Comparative Study on the Production Time of Common and Sugar-Alcohol Based DESs

Figure 18 shows that formation of a DES with microwave heating was in all cases much faster than the thermal convective heating. Thus, the formation of a DES in the microwave can save considerable time, at least in such a scale. Considering the extremely short preparation times in the microwave, that were on average 16 times faster than in the incubator, it is reasonable to think that the energy input could be lower. Furthermore, choline chloride-based DESs were prepared on average 38% faster than betaine containing ones, regardless of the method.

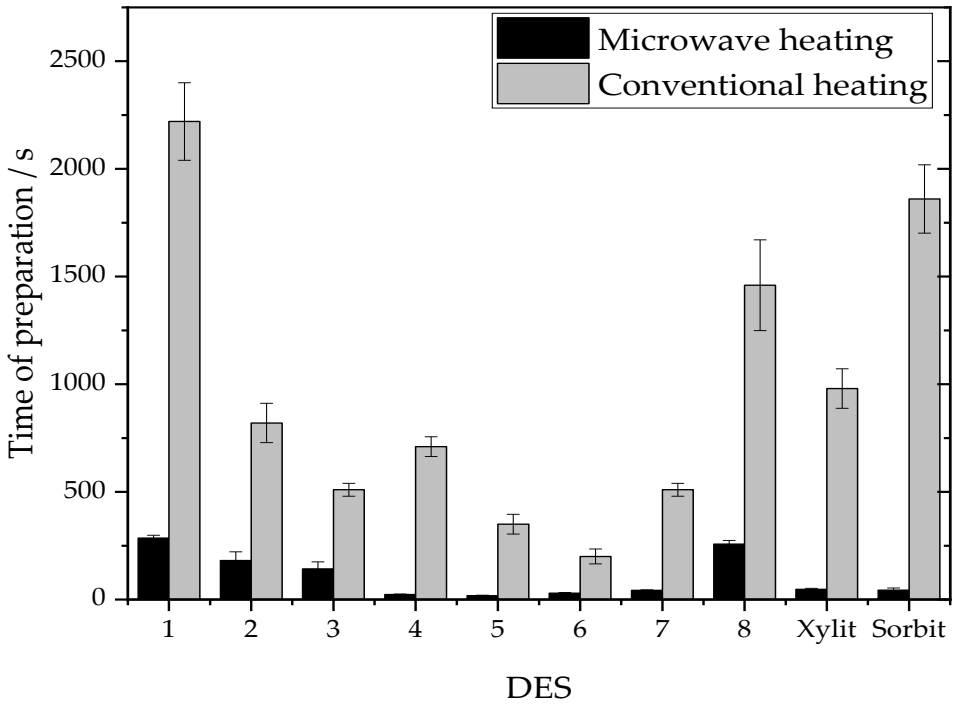


Figure 18. Preparation times of common and sugar-alcohol DESs with microwave heating and thermal heating, mean standard deviation indicates significant differences ($p < 0.05$).

Sugar-based DESs (1, 2, and 8) displayed overall longer preparation times, with either thermal or microwave heating. Such DESs are known to be difficult to prepare with conventional thermal heating and had to be produced with an adapted microwave method, since rapid heating was not possible. Adjusting the method automatically resulted in longer times to form such sensible DESs. Xylit DES and Sorbit DES, made with sugar alcohol, show drastically reduced formation times compared to the thermal heating method. Interestingly, sugar alcohol-based DESs were prepared faster than the non-reduced sugars, and observation tended to show that they were less susceptible to degradation.

Xylit DES and Sorbit DES were used to mediate the one-pot process, the sugar alcohols composing them also took part in the production of sugar alcohol esters. Next, we detail how the reaction's crude was characterized and profiled post-reaction.

4.3.2. Post-Reaction Thin Layer Chromatography (TLC) of Glycolipids- and Non Glycolipids-Containing Mixtures

The TLC of Sorbit DES was presented in **Figure 19**. Similar profiles were obtained regardless of the DES. With a standard DES, with or without lipase, only a single spot could be identified (Rf = 0.08), corresponding to the sugar alcohol. In contrast, two spots with (Rf = 0.08 and 0.34) were found in an acidified DES without lipase. In the acidified DES with lipase, the same profile was obtained, but with higher intensity of the stain corresponding to the glycolipid (Rf = 0.34). A control reaction with an acidified DES with lipase, and fatty acid methyl esters (FAMEs) from single cell oil extraction, was performed for confirmation.

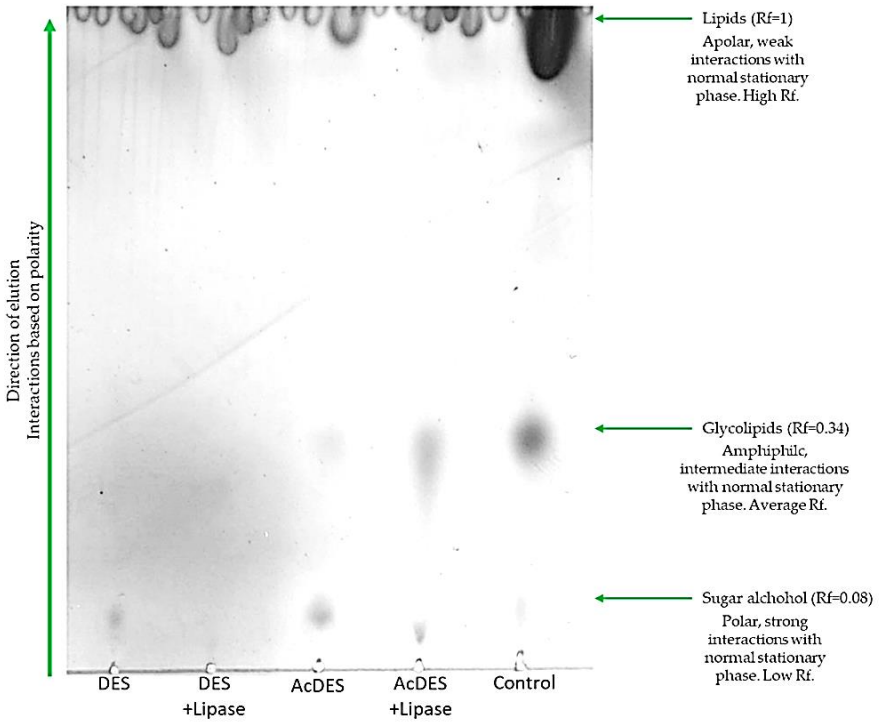


Figure 19. Thin layer chromatography of the reactions processed with microwave irradiation including a control using directly Fatty Acid Methyl Esters (FAMEs) from yeast biomass. AcDES: acidified DES; DES: standard DES.

Chromatography purification performed thereafter allowed us to isolate each fraction containing either lipids or glycolipids.

4.3.3. Comparison of Extracted Whole Cell Lipid and Esterified Fatty Acids

To be quantified, the extracted fats have been esterified into methyl esters (FAMEs), and then, injected in a gas chromatography apparatus. The mean value of lipid content for all conditions carried out on the biomass in Sorbit DES or Xylit DES was calculated.

The amounts of FAMEs, whole lipids, and glycolipids per reaction were determined and are shown in **Table 12**. The highest amount of FAMEs and whole lipids was obtained by microwave DES preparation with acidified DES, followed by the similar condition with lipase addition. In addition, the sugar alcohol in the DES did not significantly affect the lipid, and the glycolipid, yields. In comparison, lipid extraction according to Folch (FE) and standard DES, with or without lipase, were far less efficient in this regard.

Table 12. Amounts of extracted whole lipids, FAMEs, and glycolipids per 10 mL of reaction.

Condition	**DES**		**AcDES**		**DES + Lipase**		**AcDES + Lipase**		**FE**	**DT**
Sugar Alchohol	**Sorbitol**	**Xylitol**	**Sorbitol**	**Xylitol**	**Sorbitol**	**Xylitol**	**Sorbitol**	**Xylitol**	**-**	**-**
Extracted whole lipids ** (total amount (mg))	32 ± 1	50 ± 2	90 ± 1	105 ± 1.5	35 ± 1	25 ± 1	63 ± 1	68 ± 3.3	43 ± 1	-
Extracted FAMEs ** (total amount (mg))	14 ± 1	10 ± 2	45 ± 3	68 ± 3	2.4 ± 0.1	10 ± 1	40 ± 1	35 ± 1	27 ± 1	5 ± 1 ***
Glycolipid quantity (total amount (mg))	-	-	T *	T *	-	-	~15	~20	-	-

Note. *T stands for Traces meaning observation of glycolipid production could be made on TLC, but the amount was too low for chromatography purification. ** All values are given as the mean ± standard deviation of at least three independent experiments using each 400 mg of freeze-dried biomass that showed statistically significant differences, *p*-value was <0.05. *** Direct transeterification was done with 20 mg of freeze-dried biomass and only produced FAMEs.

The FAMEs and whole lipids obtained per CDW are shown in **Figure 20**. A comparison of the different lipid extraction methods showed that direct transesterification of the oleaginous biomass to fatty acid methyl esters gave the highest FAMEs yield per CDW. Extraction according to Folch was less efficient in regard of the FAMEs and whole lipids contents. In comparison to DT, acidified Xylit DES extracted up to an honorable 70% FAMEs from robust yeast cells of *S.podzolica*. Intrinsically, our DESs were not the best extraction method reported however, the subsequent interesterification of those lipids for sugar alcohol ester production made the use of these unconventional media relevant.

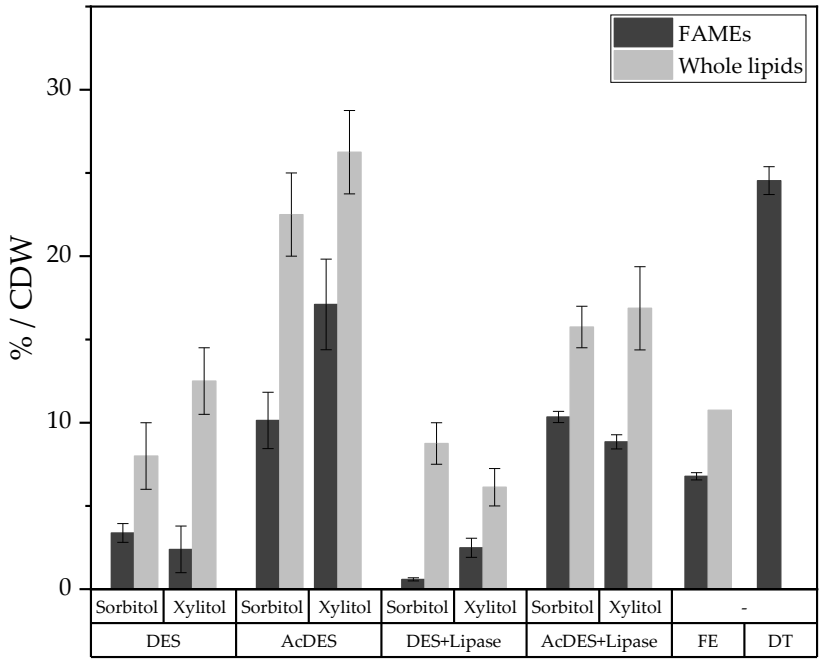


Figure 20. Fatty Acid Methyl Esters per Cell Dry Weight (FAMES / CDW) [%] for the microwave extraction of fatty acids under different reaction conditions, the Folch extraction process (FE), and the direct transesterification of the oily biomass to FAMEs (DT). Mean standard deviation indicates significant differences ($p < 0.05$).

Quantification of the various extracted fatty acids allowed then profiling of the lipid fraction.

4.3.4. Profiling of Extracted and Esterified Lipids

Lipid profiles were determined for each condition to assess if the fatty acid composition is influenced by the extraction method (**Figure 21**). When comparing the various fatty acid extraction methods, no significant differences in fatty acid profile were observed. In percentage terms, oleic acid (C18:1) with an average of 24.2% is the most common of all extraction methods, followed by palmitic acid (C16:0) with 9.0%, linoleic acid (C18:2) with 4.1%, and stearic acid (C18:0) with 2.8%.

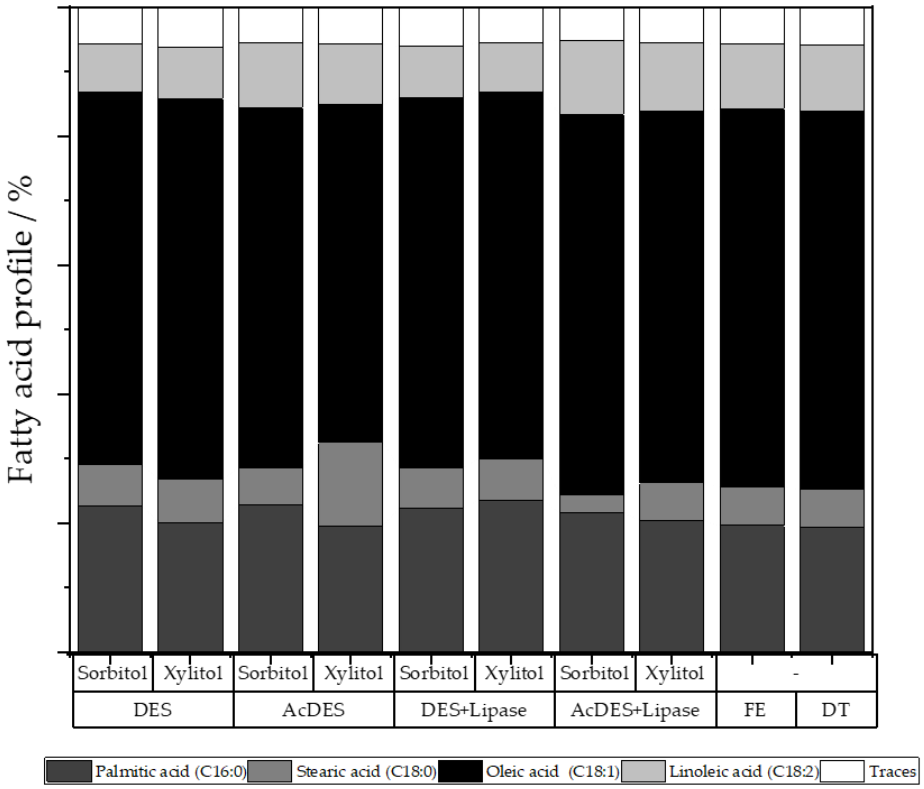


Figure 21. Distribution of fatty acids for the extraction carried out by microwave with different sugar alcohol-based DES conditions, the Folch extraction process (FE), and the direct transesterification (DT) on the oily biomass.

Nonetheless, profiling helped rationalizing and elucidating the structure of the formed sugar alcohol esters. Indeed, the most occurring lipids were more likely to react and form the targeted compounds.

4.3.5. Structural Elucidation Using Spectroscopic and Spectrometric Methods

To analyze the structure of synthesized glycolipids, ^{1}H-Nuclear Magnetic Resonance (NMR) and Mass Spectrometry (MS) were performed on the isolated glycolipid fractions. Chemical ^{1}H-NMR shifts of sugar moiety (~4.11–3.51 ppm) and fatty acid chains (~2.25–0.80 ppm) were identified. MS performed additionally allowed clear structural identification of the mono-acylated sugar alcohols. The chemical shifts were as follows:

Glycolipid fraction with Xylit DES: ^{1}H NMR (400 MHz, CD_2Cl_2) δ 4.17 (dd, J = 4.2, 2.0 Hz, 2H), 4.14 (dd, J = 3.8, 1.7 Hz, 3H), 4.15–4.08 (m, 3H), 4.12–4.06 (m, 2H), 4.02–3.92 (m, 2H), 3.90 (dd, J = 13.9, 3.5 Hz, 2H), 3.64 (dd, J = 9.6, 1.9 Hz, 1H), 2.24 (dq, J = 14.3 Hz, 4H), 1.97–1.89 (m, 2H), 1.26–1.19 (m, 38H), 0.80 (m, 9H). (**Appendix: Figure 10**).

Glycolipid fraction with Sorbit DES: ^{1}H NMR (400 MHz, CD_2Cl_2) δ 4.05 (dd, J = 12.4, 5.4 Hz, 1H), 3.75 (d, J = 6.6 Hz, 1H), 3.63–3.39 (m, 4H), 2.25 (t, J = 7.5, 2H), 1.95 (dq, J = 12.4, 6.2 Hz, 4H), 1.26–1.16 (m, 27H), 0.80 (m, 6H). (**Appendix: Figure 11**).

MS was performed with ElectroSpray Ionization (ESI) (**Appendix: Figure 12**). The results of the spectrometric analysis for the synthesis in the Xylit DES and Sorbit DES are shown in **Table 13**. Fourteen glycolipid fragments have been detected for the sorbitol-based DES. Thereof, 4 sorbitol palmitate, 4 sorbitol linoleate, 3 sorbitol stearate, and 3 sorbitol oleate. Twelve

glycolipid fragments for the xylitol-based DES were detected. Thereof, 2 xylitol palmitate, 4 xylitol linoleate, 2 xylitol stearate, and 4 xylitol oleate.

Table 13. Adducts obtained from the ElectronSpray Ionization-Quadrupole (ESI-Q) experiment for each isolated glycolipid fractions.

Xylitol-based DES Process		
Molecule	***m/z***	**Adducts**
Xylitol palmitate (XP)	391.284	$[XP+H]^+$
	409.439	$[XP+NH_4]^+$
Xylitol oleate (XO)	399.346	$[XO+H]^+$-H_2O
	417.321	$[XO+H]^+$
	434.33	$[XO+NH_4]^+$
	439.450	$[XO+Na]^+$
Xylitol Linoleate (XL)	396.427	$[XL+H]^+$-H_2O
	414.962	$[XL+H]^+$
	430.976	$[XL+NH_4]^+$
	437.434	$[XL+Na]^+$
Xylitol Stearate (XS)	401.341	$[XS+H]^+$-H_2O
	419.315	$[XS+H]^+$
Sorbitol-based DES process		
Molecule	***m/z***	**Adducts**
Sorbitol palmitate (SP)	387.235	$[SP+H]^+$-H_2O
	421.204	$[SP+H]^+$
	443.232	$[SP+Na]^+$
	437.235	$[SP+NH_4]^+$
Sorbitol oleate (SO)	393.209	$[SO+H]^+$-$3H_2O$
	447.292	$[SO+H]^+$
	465.230	$[SO+NH_4]^+$
Sorbitol linoleate (SL)	391.283	$[SL+H]^+$-$3H_2O$
	409.183	$[SL+H]^+$-$2H_2O$
	445.276	$[SL+H]^+$
	462.275	$[SL+NH_4]^+$
Sorbitol stearate (SS)	431.261	$[SS+H]^+$-H_2O
	449.271	$[SS+H]^+$
	473.344	$[SS+Na]^+$

4.4. Discussion

4.4.1. Microwave and DES Technologies: An Optimal Match?

In the present work, comparing the times required to produce common DESs with microwave and thermal heating, the efficiency of using a microwave to form these liquid mixtures was demonstrated. In theory, our approach could also significantly reduce processing time, energy costs, and equipment size compared to conventional convective or conductive thermal heating methods, but deeper investigations should be carried out to verify these claims [237–239]. Microwave assisted chemical processing requires the use of strong microwave absorbing solvents. Since the ability of some solvents, such as water, to interact in the electromagnetic field decreases sharply as temperatures rise, solvents with high thermal stability and low vapor pressure are inherently optimal [216]. DESs meet those requirements due to their polar nature and their outstanding number of hydrogen bonds. Indeed, as described by Nie *et al*., they are an ideal match for ionic conduction and dipole rotation occurring during heating. As

a result, the energy emitted by the magnetron of the microwave can be absorbed optimally by the polar and ion-containing DESs [239,240]. Thus, we conjecture that a combined effect of both greater dipole rotation and ionic conduction allowed the ultra-fast preparation of the DESs presented in our study. Within the specific framework presented in this work, one-pot microwave treatment of oleaginous biomass could then represent a more economical substitute to pre-existing, notably mechanical, preparation procedures. Finally, and according to the literature, we did not expect thermal effects to have a significant role in the acceleration of DESs preparation or in the process itself [202,241]. However, in our case, further enzymatic kinetic studies comparing, for example, the influence of classical heating and microwaves, would be needed to fully prove this last claim as the matter remains controversial and unclear [242,243].

Microwave power input is however mainly limited by thermal decomposition of the DESs components [216]. Indeed, in high temperature treatments, sugar degradation may occur through a complex series of reactions known as the Maillard reaction or caramelization. During the latter, sugar molecules are broken down, and the reaction intermediates and reactants are then polymerized [244,245]. Thus, our method used 60 °C for 72 h, mostly in opposition to the literature that reports shorter reaction times but with temperatures reaching up to 150 °C.

In general, sugar based DESs exhibit high viscosity, density, and surface tension at room temperature. Thus, it is recommended to heat these DESs for processing, to ease their handling, since viscosity decreases when temperature increases [138]. Similarly, DESs containing betaine, instead of choline chloride, also tended to show higher times and difficulty to be formed. The addition of water to promote their formation was often required, as reported for betaine mixtures with glucose, urea, and small polyol molecules [246]. Microwave heating represents therefore a viable alternative to thermal heating for an efficient and scaled-up preparation for such mixtures. Sugars, such as glucose, sucrose, and arabinose could have been also tested for the glycolipids production, but the sugar alcohols were preferred because they were less represented in the literature. Moreover, we demonstrated that in combination with choline chloride, they were among the simplest and fastest DESs to prepare using microwave irradiation. Indeed, the presence of the hydroxyl function instead of an aldehyde decreases their reactivity for polymerization. In addition, Xu *et al.* could not detect any linearity between extraction yield and polarity for the extraction of flavonoids in a DES based on sugars. However, linearity could be found with DES made of sugar alcohols [247]. Reactivity of their primary alcohols was, because of the steric hindering, similar, thus hardly influenced the results. The use of DES media for extraction is then particularly worthwhile when at least one of its component undergoes conversion, thus creating a "2-in-1" system [126].

4.4.2. Unconventional Media for Lipid Extraction and Subsequent Production of Glycolipids

In this work, lipid extraction was achieved by treating the biomass with a combination of microwave, DES, and enzyme technologies that were individually reported as possible cell disruption methods [232]. Our designed one-pot extraction of lipids and production of mono-acylated sugar alcohol esters drastically reduced steps number. However, pretreatment of biomass by freeze-drying was still necessary, because moisture considerably reduced the yield of transesterification [248,249]. Indeed, moisture content of oleaginous microorganisms was over 80% [250]. In this work, highest lipid content could be measured with use of an acidified DES, followed by an acidified DES with addition of lipase. Since glycolipids were

predominantly formed in an acidified DES with lipase, slightly less lipids have been measured as FAMEs. According to Gorte *et al*., it has been shown that by using acid instead of base, significantly more fatty acids could be obtained in the direct transesterification of the biomass. Yeast cell wall structure usually consists of two layers, including an inner layer with polysaccharides and an outer layer with glycoproteins covalently bound to the inner layer [251]. Thus, *S. podzolica* cells were very robust and needed harsh conditions for lipid extraction. That is why, in previous reports, mechanical methods such as high-pressure homogenizer and ball milling could recover higher yields of lipid per CDW and appeared more efficient that physical digestion with acidified DES.

Khoomrung *et al*. demonstrated the potential of a microwave-assisted technique for the rapid lipid extraction from yeast cells [252]. In addition, it has been shown that microwave pretreatment was one the most efficient methods among sonication, bead milling, and osmotic shock to extract lipids from oleaginous biomasses such as algae [253]. Carboxylic acid-based DESs seemed more suitable for the lipid extraction of oleaginous biomass, in comparison to sugar or polyol-based DESs [254]. This could be correlated to our results, which highlights that the sugar alcohol-based DES extracted significantly more lipids when acidified.

Lipase did not significantly influence the lipid extraction efficiency, since almost identical amounts of lipid contents were obtained in a standard DES, with or without lipase. However, lipase seemed to promote the glycolipid synthesis (**Figure 19**). An interesting aspect was the positive influence of HCl on the enzyme activity. It is known that Novozym 435® is stable over a relatively wide pH range, especially in the alkaline one. Here, we demonstrated that glycolipids could be formed in an acidified DES with lipase. Thus, we speculated that inactivation of the lipase was limited in the presence of 0.1 M of HCl in DES, since it was shown that Novozym 435® retained activity up to 0.3 M of acetic acid [255]. We conjecture that due to the high viscosity and low water activity/content of DESs [256], lipase activity was predominant as only a limited quantity of media could accumulate inside the porous acrylic carrier (Lewatit 1600) of Novozym 435®. Thus, the inactivation of the biocatalyst was rather minimal [145]. The conversion could partly occur due to acid or auto-catalysis, but based on our results, it is considered negligible [257–259]. ILs showed great efficiency toward lipid extraction as well, but imidazolium-based ones inhibited lipase activity [260,261]. Therefore, DESs represent a greener and simpler alternative in which biocatalysis can be performed [262,263]. Their association with microwave irradiation appeared interesting to form an effective duo using relatively mild conditions with overall low energy consumption [264].

During the flash chromatography purification, the glycolipid peak was only observed in the acidified DES with lipase, most likely because its concentration was too low to be detected by the light scattering detector (**Appendix: Figure 13**). Glycolipids were therefore obtained in quantities that allow flash chromatography purification when both acidification and the enzyme were combined.

Based on our "2-in-1" deep eutectic system [126], with the microwave-assisted procedure, glycolipids can be tailor-made based on any sugar- (or polyol)-based DES with any lipid-containing biomass [265]. The current work using *S. podzolica* biomass made it possible to obtain long-chain glycolipids with no prior modification of the lipids (e.g., ethylation, methylation, or vinylation) [128]. These sugar esters find application in various fields, such as in the cosmetics industry (e.g., rhamnolipids, sophorolipids, and mannosylerythritol lipids)

and in the pharmaceutical industry (e.g., mannosylerythritol lipids) [7]. Insects such as *Hermetia illucens* also represent an interesting source of oleaginous biomass as they are more susceptible to provide short-chain lipids, notably lauric acid, and myristic acid [266,267]. Thus, by using such a biomass, short-chained glycolipids could be formed. The production of laurate sugar esters raises interest, especially in the pharmaceutical industry where they are notably used to form drug delivery systems [134,268]. The properties of the glycolipids can be influenced not only by chain length but also by the ramifications and degree of unsaturation [269,270]. Furthermore, use of oleaginous biomass allows esterification of branched and unbranched fatty acids, that have different chain lengths and contain one or several alkene groups that represent gateways for further chemoenzymatic reactions [117].

4.5. Conclusions

In this chapter, we demonstrated a proof of concept of the lipid extraction from oleaginous biomass and the subsequent glycolipids formation using lipase-catalyzed reaction with microwave-assisted processing in acidified DES. The medium made of choline chloride and sugar alcohols was prepared, among other more common DESs, in a matter of seconds using microwave technology, highlighting a technological match. In such "2-in-1" system, any combination of sugars and lipids is virtually possible to prepare tailor-made glycolipids. Indeed, other oleaginous biomasses for the synthesis of glycolipids could be considered. With the help of the developed robust reaction system combined with a proper identification strategy, compounds were isolated and characterized. In addition, screening for potential bioactivity of the DES containing mixtures or isolated compounds would be an interesting follow-up.

However, only a limited amount of glycolipid was obtained making optimization required. It is therefore important to investigate higher temperatures, different lipase formulations, and develop kinetic determination methods to allow an optimized scale-up of our process. In addition, chloroform and methanol were used as solvents for the purification of the glycolipids by flash chromatography. The replacement of the latter by halogen-free, more environmentally friendly solvents, such as hexane and ethyl acetate, or developing alternative methods to get rid of chromatographic purification should be considered.

The acidification of DESs to promote the lipase-catalyzed reaction must be understood in the future. Finding how much glycolipid can be produced by the specific activity of the lipase and how these conditions affect the overall activity and stability of the lipase formulation, as well as the recyclability of the biocatalyst, should all be investigated.

5. Enhanced Bioactivity of Tailor-Made Glycolipid Enriched Manuka Honey

This chapter is partly based on the publication draft:

Enhanced Bioactivity of Tailor-Made Glycolipid Enriched Manuka Honey
André Delavault, Ahmed E. Zoheir, Delphine Muller, Kersten S. Rabe, Katrin Ochsenreither, Jens Rudat and Christoph Syldatk
To be submitted

Authors Contribution:

André Delavault: conceptualization, methodology, formal analysis, investigation, writing of the original draft, writing of the reviews, and editing

Ahmed E. Zoheir: conceptualization, methodology, formal analysis, and investigation

Delphine Muller: methodology and investigation,

Kersten S. Rabe: supervision and funding acquisition

Katrin Ochsenreither: methodology and writing of the reviews, and editing

Jens Rudat: formal analysis, writing of the original draft and writing of the reviews

Christoph Syldatk: funding acquisition and supervision

5.1. Introduction

Since thousands of years, honey has been employed for its medicinal properties, notably as a wound dressing [271]. From a physico-chemical point of view, it is a viscous, supersaturated sugar solution derived from the nectar, gathered and modified by the honeybee and it is possibly one of the few natural Deep Eutectic Solvent (NADES) [256]. This term describes a liquid phase obtained by combining Hydrogen Bond Donors (HBDs) with high melting points, which are abundant in nature such as carboxylic acids, sugars and amides. Their high biodegradability and high recyclability combined with a fairly low water content make them a powerful tool for biocatalysis [121]. As a result, honey is a media that presents interesting properties for chemical and biological purposes.

When pasture honeys are diluted down to 25% of the original volume concentration, they tend to lose their antibacterial activity, most probably due to dilution of the active compounds such as flavonoids, neutralization of pH and decrease of osmolarity [272,273]. In a practical case, dilution of honey, e.g., used as wound gel, can occur when the wound exudates. The "ideal medical honey" would have a relatively strong and rapid bactericidal effect compared to standard honeys. [274]. In other terms, the antibacterial efficacy would rely on compounds that are effective even upon dilution. In this aim a good candidate arises: the standardized medical-grade Manuka honey (MH) which exhibits high levels of MGO (Methylglyoxal) and shows intrinsic antibacterial activity up to 30% dilution [275]. However, MGO belong to the α-ketoaldehyde family, a special class of 1,2-dicarbonyl compound, which is highly reactive and as a result non-stable [276,277]. The aim is to strengthen and extend in time the overall antibacterial effect by enriching the media with more stable bioactive compounds that are also effective upon dilution.

Using surfactants for this purpose seems promising, Kwakman *et al.* tried this principle and obtained convincing results [278]. Indeed, their strategy allowed a more rapid bactericidal effect against antibiotic resistant pathogens (~2 hours) compared to honey alone. However, they tested two surface-active peptides consisting of long amino acid chains (BP2 and LL37, respectively 19-mer and 37-mer) which were synthetized using solid phase and protection/deprotection technique. Either sequential or convergent, this synthesis method is time costly and expensive, making it less relevant for a larger scale process as honey and lipase formulations are produced on a multi-ton scale at food grade. Pöhnlein *et al.* demonstrated the enzymatic synthesis of glycolipids, a class of surfactants, with DES acting as solvent and substrate at the same time [119]. More recently, Siebenhaller *et al.* successfully applied this approach using honey as a solvent and its components, essentially fructose and glucose, as substrates [279]. This synthesis relies on the use of the immobilized lipase from *C. antarctica* (iCalB or Novozym 435®) for the transesterification between vinyl esters and sugars naturally present in honey. Vinyl esters are substrates of choice for enzymatic transesterifications thanks to the vinylic function representing an electrophilic site that offers irreversible and enantioselective acylation of primary hydroxyl groups [128]. The resulting vinyl alcohol as a side product undergoes tautomerization that produces acetaldehyde, a volatile compound that evaporates and thus, shifts the reaction equilibrium toward ester formation as depicted in the **Figure 22** [280].

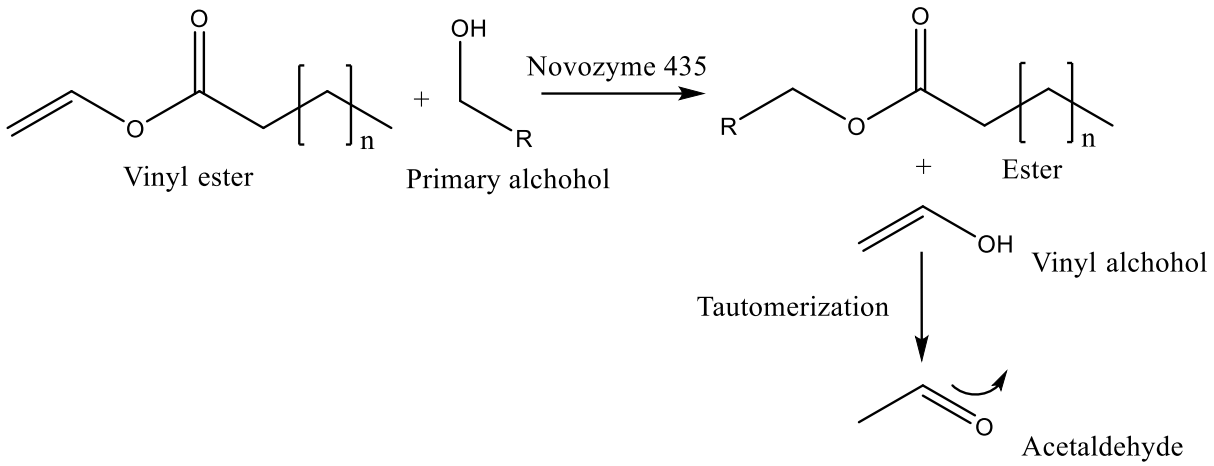


Figure 22. Immobilized lipase catalyzed transesterification between primary alcohol from honey sugars (R= glucose, fructose) and vinyl esters leads to newly formed esters (n=6,7,9,11,13).

Products of this synthesis, such as fructose laurate, are known to possess antibacterial effects [281]. But other derivatives such as octanoate, decanoate, laurate and myristate sugar esters have not yet been tested for bioactivity in combination with honey. Herein, these reactions are carried out in one of the most efficient honey against pathogens according to literature: MH from New-Zealand derived from the Manuka tree (*Leptospermum scoparium*) [282]. Honey as pasture or wound gel might be a promising alternative strategy to treat surficial wound infections by antibiotic-resistant pathogens [283], since the resistance is increasing worldwide [284] and very few new antibiotics are being developed [285].

In this study, a first qualitative screening with different microorganisms was performed to determine which kinds of bacteria or yeasts were susceptible to honey mixtures enriched with different sugar esters. Those microorganisms which are MRSA, *Bacillus subtilis*, *Candida bombicola*, *Escherichia coli* and *Pseudomonas putida* represent a versatile testing pool with yeast, Gram– and Gram+ microorganisms to determine what could be the targets of such mixtures from a pharmaceutical point of view.

Recently, Zoheir *et al*. [286] developed a new biosensor system for multiple stress sensing which allows monitoring of internal and external stresses in *E. coli* cells by using a three-colored fluorescent protein combination. These proteins function throughout three principal stress response mechanisms that are the RpoS pathway (starvation) [287], RpoH (heat-shock) [288] and SOS (DNA-damage) [289], all of which are controlled by specific promoters. This system called RGB-S reporter allows a real time and simultaneous analysis of the stress responses in *E. coli* by microplate reading equipped with fluorescence detection. This biosensor system has been used in the current work to characterize the bioactivity of the glycolipid enriched honey mixtures. Thus, giving a deeper insight on how a pathogen such as *E. coli* is affected by enriched honey mixtures. Quantitative agar well diffusion experiments also permitted to link measurable inhibition of biofilms on agar plates and multi-stress biosensing. In the end, the most efficient of these mixtures containing octanoate sugar esters (GOH) was compared to the non-enhanced honey to determine upon which concentration glycolipids permit enhancement of the bioactivity on agar plate.

5.2. Materials and Methods

5.2.1. Chemicals

Lipase B from *Candida antarctica* immobilized on acrylic resin (Novozym 435®), Mueller Hinton Broth, kanamycin and LB broth were purchased from Merck (Germany). Commercially available Manuka Honey MGO 550+ (MH, Manuka Health, New Zealand) was purchased from Real GmbH (Germany) and used as substrate. All fatty acid vinyl esters were acquired from Tokyo Chemical Industry Co., Ltd. (TCI-Europe, Belgium). Ultrapure water used for dilution of the MH and honey mixtures was obtained with a Purelab flex purification system (Ransbach-Baumbach, Germany). European agar was obtained from Becton Dickinson (Le Pont de Claix, France). Rhamnolipid standard mixture JBR599 was purchased from Jeneil Biosurfactant Co. (Saukville, USA). If not stated otherwise, all other chemicals were purchased from Carl Roth (Germany).

5.2.2. Microorganisms

Microbicidal tests were assessed against methicillin-resistant *S. aureus (*MRSA DSM 11729*), B. subtilis* (ATCC 21332), *C. bombicola* (ATCC 22214), *E. coli* (K12 DSM 498) and *P. putida* (DSM 5235). For the stress sensing assay, RGB-S reporter was assembled as described by Zoheir *et al.* [286] on chemically competent cells of wildtype *E. coli* K12 MG1655 DSMZ 18039 (DSMZ GmbH, Germany) that were transformed with the RGB-S reporter (designated as sensor strain) and plated on Luria-Bertani (LB; peptone 10 g/L, yeast extract 5 g/L, sodium chloride 5 g/L; for solid medium 15 g/L agar was added) agar plates supplemented with 50 mg/L kanamycin (designated as LB+kan).

5.2.3. Preparation of Glycolipid Enriched Manuka Honey

The enzymatic synthesis of glycolipids in Manuka honey (MH) is based on Siebenhaller *et al*. [279] with slight modifications as follows: 200 mg of Novozym 435®, $1.03x10^{-3}$ mmol of pure fatty acid vinyl ester (vinyl octanoate, vinyl decanoate, vinyl laurate, vinyl myristate and vinyl palmitate) and 2.5 mL of MH were filled in a 5 mL Eppendorf tube. After 30 s vortex homogenization, the reaction was carried out in a rotator mixer with U2 program at 50 rpm (neoLab, Germany) at 50°C for 48 h. Five different glycolipid enriched MH mixtures were formed containing sugar esters of octanoate (GOH), decanoate (GDH), laurate (GLH), myristate (GMH) and palmitate (GPH). An additional mixture which did not contain enzyme but only $1.03x10^{-3}$ mmol of vinyl octanoate (MHWE) was also incorporated to the antibacterial tests as comparison.

5.2.4. Extraction and Detection of Glycolipids via Thin Layer Chromatography (TLC)

Synthesized glycolipids were extracted from honey media by addition of 2 mL of warm water and homogenization of the resulting mixture. After addition of 3.5 mL ethyl acetate and vortexing for 30 s, a glycolipid-containing organic phase was formed and further used for TLC analysis as follows. 10 µL of the previously extracted organic phase were spotted onto a silica plate (Alugram SIL G, 60 Å, Macherey-Nagel GmbH & Co., KG, Germany). The eluent consisted of chloroform: methanol: acetic acid (65:15:2 v/v) [119]. After elution, TLC plate was dipped into anisaldehyde: sulfuric acid: acetic acid (0.5:1:100 v/v) dying solution and subsequently revealed with a heat gun.

5.2.5. Susceptibility Test

Susceptibility test with the different microorganisms and glycolipid enriched mixtures was qualitatively assessed using an agar well diffusion assay according to Mavric *et al.* [275]. The microorganisms were pre-cultivated overnight at 37°C (MRSA, *E. coli* and *P. putida*) or 30°C (MRSA, *B. subtilis* and *C. bombicola*) in 100 mL flasks containing 10 mL of nutrient broth according to [290]. Afterwards 100 µL of the undiluted cultures (0.4 OD_{600}) were spread on plates containing solidified nutrient medium. Wells 8 mm in diameter (150 µL capacity) were punctured into the surface of the agar medium. 120 µL of solutions of the enriched honey mixtures, MH or MH plus fatty acid containing no enzyme were placed into the wells. Plates were incubated at 37°C or 30°C depending on the microorganisms and observation on the inhibition zone was made after 24h.

5.2.6. Stress Assay Protocol

For the stress assay, an aliquot of the seedbank was used to inoculate two independent cultures (Cult. 1 & Cult. 2) each in 5 mL LB+kan broth and incubated overnight at 180 rpm and 37 °C. The next day, cultures were diluted to 1:250 using fresh LB+kan and incubated again at the same conditions for 5-6 hours. After reaching adequate optical density (OD_{600}= 0.8), the cultures were diluted using fresh LB+kan to adjust the OD_{600} to 0.4, which is 2X of the final cell density in the assay plate. Honey mixtures were diluted in LB+kan to 2X of the final required concentration. The stress treatment started by adding 250 µL diluted sensor strain culture to 250 µL LB-stress mixture (or LB containing no honey mixtures as the control culture) to form 500 µL total volume, which had a final cell density of OD_{600} 0.2 and 1X stressor concentration. Upon mixing, each of the two cultures (Cult. 1 & Cult. 2) were then distributed in triplicates in 96-microwell plate, each well containing 150 µL. In total, six independent biological replicates were analyzed for every honey mixture concentration unless indicated otherwise. Tested concentrations of MH, MHWE, GOH, GDH, GLH and GMH were 0, 0.1, 0.2, 0.4, 0.8, 1.2 and 1.6 % (v/v). The microtiter plate was covered by a fluorescence-compatible transparent film (Lab Logistics Group Inc.) to prevent culture evaporation and incubated in a Synergy H1 microplate reader (BioTek Inc.) with continuous orbital shaking (282 rpm, 3 mm) at 37 °C and measuring the optical density (OD_{600}) and the three fluorescence readouts corresponding to the different types of stress. For the fluorescence readouts, respectively excitation and emission peaks correspond to the following wavelengths: Red Fluorescent Protein (RFP, physiological stress): 571 nm, 607 nm; Blue Fluorescent Protein (BFP, cytotoxic stress): 400 nm, 454nm; Green Fluorescent Protein (GFP, genotoxic stress): 483 nm, 511 nm.

5.2.7. Assessment of Antibacterial Activity

Antibacterial activity of GOH and MH was quantitatively assessed using an agar well diffusion assay according to Patton *et al*. [291]. E. coli was pre-cultivated overnight at 37°C in 100 mL flasks containing 10 mL of Mueller Hinton Broth (MHB) medium. Afterwards 100 µL of the cultures (0.1 OD_{600}) were spread on plates containing solidified MHB agar. Wells 15 mm in diameter (200 µL capacity) were drilled in the broth-agar medium. 150 µL of solutions of the GOH and MH diluted to concentrations ranging from 0% being the control well to 50% in ultrapure water were placed into the wells. Plates were incubated at 37°C for 24h. The zones of inhibition were measured using pictures taken into a FAS Digi Imaging System (FastGene) with a GF-7 digital camera (Panasonic). Measurements were then made with ImageJ software.

5.3. Results

5.3.1. Glycolipid Synthesis in Honey

Glycolipids were synthesized in Manuka honey by adding different fatty acid vinyl esters (octanoate (C8, GOH), decanoate (C10, GDH), laurate (C12, GLH), myristate (C14, GMH) and palmitate (C16, GPH)) and immobilized lipase (iCalB). Product formation was qualitatively assessed by thin layer chromatography (TLC). **Figure 23** shows similar TLC profiles between GOH, GDH, GLH, GMH and GPH mixtures. Sugars esters appeared with an average retention factor Rf=0.65 after dying with anisaldehyde solution as previous work reports from Siebenhaller *et al*. [279]. Highly polar compounds such as sugars in this case have more interaction with the silica coated layer therefore giving a low retention factor (Rf=0.12). Unreacted free fatty acids that are hydrophobic present low interaction with the stationary phase and move with the elution front "pushing" them on the very top of the TLC. Glycolipid synthesis was successful with all applied vinyl fatty acids into Manuka Honey. To firmly support the last claim, a standard that acts as a control was also spotted on the same TLC. This standard (Std) is a commercially available rhamnolipid mixture diluted at 10 mg/mL in ethyl acetate which displays glycolipid stain at Rf=0.68 on normal phase thin layer chromatography. The relative observable intensity of the TLC stains indicates that in each mixture glycolipids were produced in the same range of concentrations. MHWE serves as a negative control for the glycolipid synthesis since no enzyme was introduced in this condition, only unreacted vinyl octanoate is present. It shows no characteristic spot of glycolipids affirming that specific conversion of substrates into glycolipids is due to the enzyme.

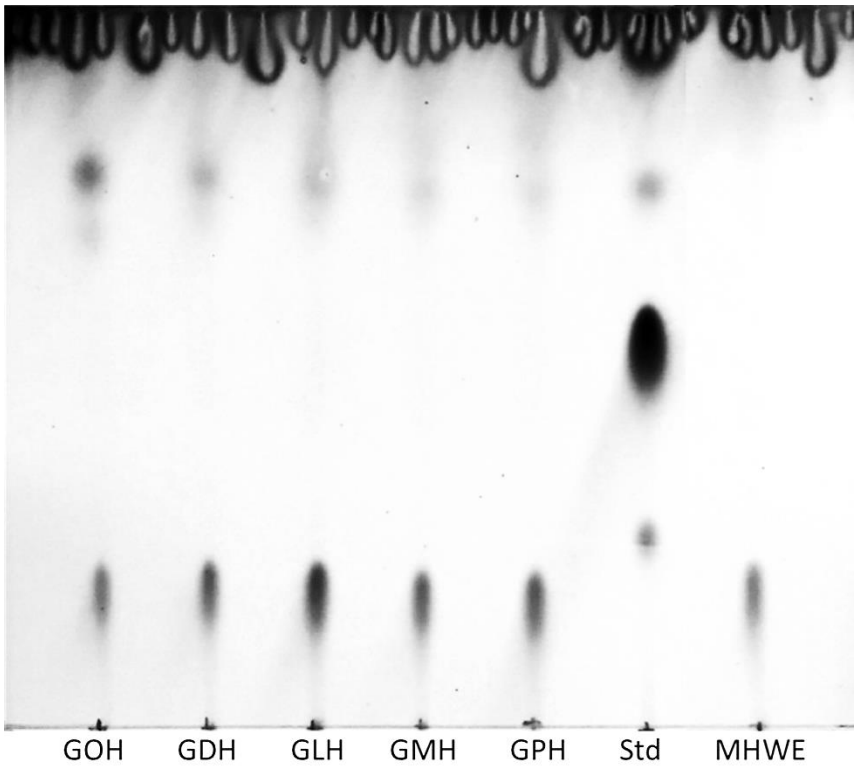


Figure 23. Visualization of synthesized glycolipids in Manuka Honey after dying with an anisaldehyde solution. 10 μL of extracted honey mixtures and 4 μL of the standard were spotted on the TLC plate. Std, intern laboratory rhamnolipid standard; GOH, glycolipid octanoate honey; GLH, glycolipid decanoate honey; GLH, glycolipid laurate honey; GMH, glycolipid myristate honey; GPH, glycolipid palmitate honey; MHWE, Manuka honey without enzyme.

5.3.2. Microorganisms Susceptibility Test

This qualitative antimicrobial test was performed with the aim of a pre-selection of glycolipid enriched honey mixtures using observable inhibition as an orientation. It oriented the work toward the types of microorganisms that show susceptibility to the different glycolipid enriched MH mixtures. The latter containing palmitate (GPH) was also tested with this assay, but results showed very poor efficacy and therefore was non-relevant for the current work so finally left aside. **Table 14** shows that every honey-based mixture mentioned above present bioactivity towards the different microorganisms tested. Gram+ and Gram– bacteria being seemingly sensible, but the yeast species (*C. bombicola*) displayed a very particular susceptibility to the GOH mixture (**Figure 24**). Slight or no effect is observable for MH, MHWE, GDH, GMH and GLH. GOH showed very clear and large inhibition zones of the biofilm on all microorganisms that have been tested. *E. coli* and MRSA showed particularly high susceptibility to all honey mixtures, glycolipid enriched or not. Thus, highlighting the tendency of every Gram+ microorganisms to be sensible to MH mixtures. Contrarily, Gram– bacteria and the yeast displayed acute sensibility to mixtures enriched with sugar esters.

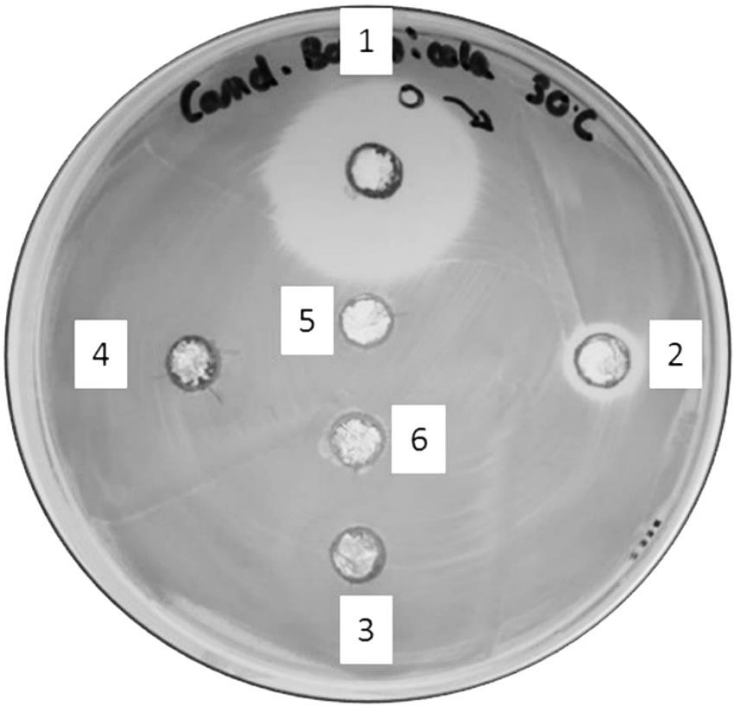


Figure 24. Qualitative agar well diffusion assay (Candida bombicola). Every honey mixture was incorporated undiluted in the wells. Well 1, GOH; Well 2, GDH; Well 3, GLH; Well 4, GMH; Well 5, Manuka Honey; Well 6, Manuka Honey mixture with 0,31 mmol/mL vinyl octanoate and no enzyme (MHWE).

As *E. coli* was more inhibited by all glycolipid enriched honey mixtures than with MH alone, a reporter strain was applied to characterize the type of stress which is exerted by the respective glycolipid enriched honey mixture.

Table 14. Qualitative agar well diffusion assay highlighting microorganism susceptibility. System allows relative comparison with mixture efficacies using a pluses system. It categorizes the effect based on the diameters of the inhibition zones: +++, diameters ≥ 29 mm. ++, diameters < 29 mm and ≥ 19 mm. +, diameters < 19 mm and > 0 mm. 0, no observable effect. MH, Manuka Honey; MHWE, Manuka honey and unreacted vinyl octanoate (negative control for glycolipid synthesis: approach without enzyme); GOH, glycolipid octanoate honey; GLH, glycolipid decanoate honey; GLH, glycolipid laurate honey; GMH, glycolipid myristate honey.

Microorganisms/Cell type	MH	MHWE	GOH	GDH	GLH	GMH
MRSA/Gram+	++	++	++	++	+	+
B. subtilis/Gram+	+++	+++	+++	+++	+++	+++
C. bombicola/Yeast	0	0	+++	+	0	0
E. coli/Gram–	++	++	+++	+++	+++	+++
P. putida/Gram–	+	+	++	+	+	+

5.3.3. Whole Cell Multi-Stress Biosensing and Bactericidial Activity in Broth Dilution

E. coli cells used as stress biosensors were incubated 24h with glycolipid enriched honey mixtures at 37°C to characterize the apparent bioactivity. In this experiment three different types of stress reporters allow characterization of the bioactivity. Physiological, cytotoxic and genotoxic stress were simultaneously detected *via* fluorescent microplate reading due to three fluorescent proteins giving signals called RFP, BFP and GFP, respectively. Those signals were normalized to the OD_{600} of *E. coli* to get a signal that is specific to the biomass. **Figure 25** displays measures for the different honey mixtures at 1.6% (v/v) which is the most representative of all tested concentrations according to **Table 15** that gathers measures for all mixtures after 20 hours of incubation.

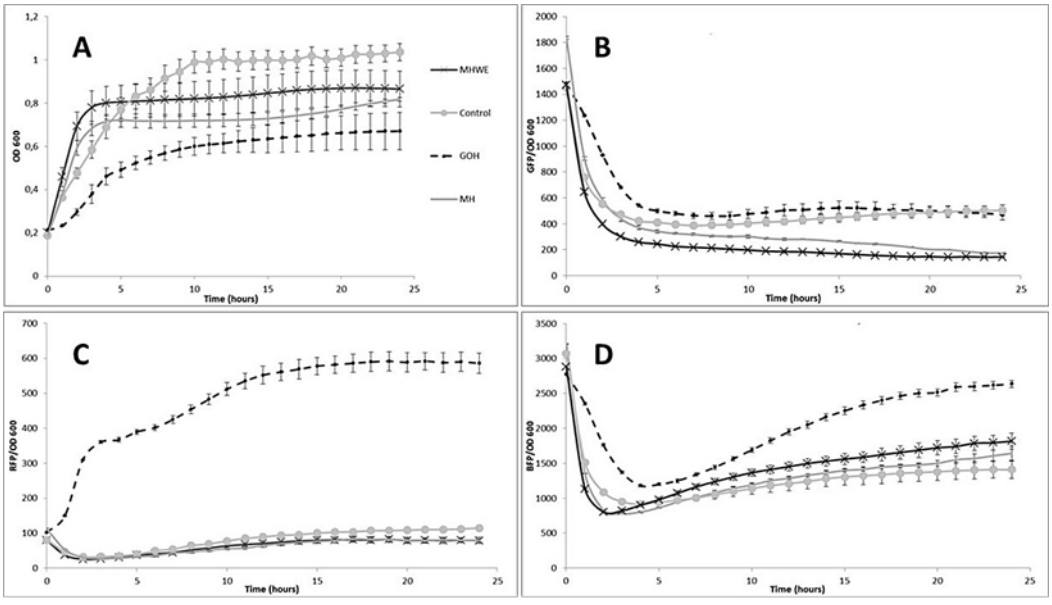


Figure 25. Biosensor based stress assay using designed E. coli strain. The bioactivity and the type of stress induced by the different honey mixtures were assessed with a multi-stress whole-cell biosensor set-up called RGB-S reporter. The readout displays: (A), OD_{600} (culture growth). (B), GFP (genotoxic stress). (C), RFP (physiological stress). (D), BFP (cytotoxic stress). Signals are normalized to the OD_{600} of the biosensor giving specific signals for each type of stress. 24 hours kinetic with one measure per hour at 1.6 % (v/v) displaying bioactivity of MH, MHWE and GOH. Measurements were done in a double triplicate using two different cell cultures.

Table 15. Stress assay reading at 20 hours displaying bioactivity of MH, MHWE, GOH, GDH, GLH and GMH at 0, 0.1, 0.2, 0.4, 0.8, 1.2 and 1.6 % (v/v).

Mixture	Concentration % (v/v)	OD_{600}	Specific RFP	Specific BFP	Specific GFP
Control	0	1.01 ± 0.04	108 ± 3	1474 ± 223	694 ± 87
MH	0.1	1.02 ± 0.01	109 ± 12	1352 ± 65	239 ± 26
	0.2	0.74 ± 0.04	96 ± 20	1309 ± 55	234 ± 9
	0.4	0.74 ± 0.04	74 ± 11	1287 ± 36	233 ± 8
	0.8	0.76 ± 0.02	65 ± 13	1328 ± 33	185 ± 8
	1.2	0.78 ± 0.02	69 ± 14	1361 ± 37	190 ± 10
	1.6	0.77 ± 0.02	79 ± 6	1499 ± 40	202 ± 4
MHWE	0.1	1.03 ± 0.04	102 ± 18	1614 ± 155	279 ± 37
	0.2	0.85 ± 0.11	89 ± 9	1448 ± 80	180 ± 7
	0.4	0.84 ± 0.10	89 ± 7	1447 ± 50	173 ± 5
	0.8	0.82 ± 0.11	74 ± 8	1770 ± 129	148 ± 7
	1.2	0.84 ± 0.07	78 ± 8	1683 ± 83	149 ± 4
	1.6	0.87 ± 0.08	79 ± 9	1721 ± 101	147 ± 8
GOH	0.1	0.81 ± 0.04	126 ± 11	1449 ± 76	265 ± 17
	0.2	0.78 ± 0.09	121 ± 22	1169 ± 19	216 ± 5
	0.4	0.64 ± 0.02	127 ± 14	1360 ± 26	242 ± 7
	0.8	0.60 ± 0.02	220 ± 15	1578 ± 11	303 ± 4
	1.2	0.69 ± 0.02	582 ± 47	1855 ± 95	313 ± 11
	1.6	0.66 ± 0.08	589 ± 28	2512 ± 51	500 ± 38
GDH	0.1	1.00 ± 0.02	79 ± 13	1429 ± 129	284 ± 27
	0.2	0.93 ± 0.03	91 ± 21	1425 ± 187	176 ± 4
	0.4	0.80 ± 0.03	79 ± 7	1398 ± 109	160 ± 6
	0.8	0.85 ± 0.08	80 ± 13	1674 ± 359	158 ± 5
	1.2	1.10 ± 0.07	76 ± 18	1507 ± 380	132 ± 6
	1.6	1.19 ± 0.04	75 ± 13	1269 ± 187	123 ± 5
GLH	0.1	1.04 ± 0.01	206 ± 10	1335 ± 34	283 ± 31
	0.2	1.01 ± 0.03	178 ± 16	1331 ± 61	168 ± 13
	0.4	0.81 ± 0.01	83 ± 5	1174 ± 46	185 ± 5
	0.8	1.00 ± 0.02	99 ± 9	1054 ± 20	132 ± 2
	1.2	1.07 ± 0.03	99 ± 9	1064 ± 42	126 ± 8
	1.6	1.11 ± 0.02	106 ± 7	1101 ± 37	127 ± 5
GMH	0.1	1.17 ± 0.03	142 ± 39	1806 ± 247	337 ± 100
	0.2	0.78 ± 0.02	146 ± 20	1739 ± 29	219 ± 4
	0.4	0.72 ± 0.03	140 ± 27	1803 ± 78	227 ± 6
	0.8	0.80 ± 0.05	103 ± 9	1623 ± 91	189 ± 5
	1.2	0.84 ± 0.05	123 ± 29	1791 ± 149	196 ± 7
	1.6	0.91 ± 0.08	157 ± 24	1887 ± 268	201 ± 7

Note. Standard deviation (±) is expressed from two triplicates obtained from two independent cell cultures.

Stress assay showed a 6-fold increase for the physiological stress, a 2.5-fold increase of cytotoxic stress and no change in genotoxicity when MH is enriched with octanoate sugar esters (**Figure 25B, C and D)**. In comparison, other mixtures like GDH, GLH and GMH have very low or essentially no effect on *E. coli*´s growth reflected by almost negligible stress signals. Moreover, the growth curve displays the rapid effect of GOH compared to the other mixtures with a lag phase of the growing *E. coli* culture starting at early incubation and lasting for 4 hours (**Figure 25A**). Thus, highlighting the fact that, over an extended period, GOH possesses the most efficient and rapid bioactivity of all mixtures tested. MHWE which consisted of the same mixture of MH and vinyl octanoate, except no enzyme was included, showed an obviously smaller effect that is like the control.

5.3.4. Whole Cell Multi-Stress Biosensing and Bactericidial Activity in Broth Dilution

E. coli was also used for an agar well diffusion assay aimed for a quantitative experiment in which inhibition diameters of different concentrations have been measured. Previous results oriented us to compare the bioactivity of MH and GOH in a bactericidal assay upon same dilutions. Concentrations ranging from 0 to 50% (v/v) with 10% steps were tested. Each condition was repeated throughout three wells. Same dilution ranges were compared across different agar plates. No bioactivity was observed with MH at concentrations lower than 40% (v/v) whereas GOH showed direct inhibition of the microorganism at a concentration of 20 % (v/v). Diameters of the inhibition circles are reported in **Table 15**. When concentration reaches 40% (v/v) inhibitory potential of GOH and MH appears to be identical. Thus, the GOH mixture obviously exhibits antimicrobial effects at lower concentrations than pure MH.

Table 16. Inhibition zones measured for different concentrations of MH and MGO using *E. coli*. Wells were done in triplicates across different agar plates.

	Concentration	Well 1	Well 2	Well 3	Average	Standard deviation
Control	0%	0	0	0	0	0
	10%	0	0	0	0	0
	20%	1.72	1.85	1.961	1.85	0.12
GOH	30%	1,93	1,90	1,955	1.93	0.03
	40%	2.13	2.18	2.21	2.17	0.04
	50%	2.23	2.35	2.24	2.27	0.06
	10%	0	0	0	0	0
	20%	0	0	0	0	0
MH	30%	0	0	0	0	0
	40%	2.00	2.12	2.23	2.11	0.14
	50%	2.13	2.28	2.54	2.32	0.21

5.4. Discussion

5.4.1. Impact of the Process on Endogenous and Exogenous Compounds in Manuka Honey

The method used in the current work for glycolipid production in honey was validated in a previous publication from Siebenhaller *et al.* [279]. Glycolipids coming from the transesterification of vinyl octanoate (C8), vinyl decanoate (C10), vinyl laurate (C12) and vinyl palmitate (C16) with sugars from honey and agave syrup were successfully identified and characterized using spectrometric methods. Given those previous results it is assumed that all vinyl esters used represent good substrates for Novozym 435® to synthetize the corresponding sugar esters. Slight changes were brought to the present process and even though both synthesis methods are still very similar, those changes must be considered. First off, a different honey was used, in the first research article a standard European flower honey was taken whereas in the current work MH was chosen and used as a substrate. Second off, the novelty brought herein is the use of vinyl myristate (C14) instead of the palmitate vinyl ester (C16). MH was chosen as a substrate and solvent for carrying this reaction due to its well established bioactivity that is higher than other honeys and displays efficacy against several pathogens [292–294]. The aim herein was firsthand to select the honey presenting the best bioactivity according to literature and secondhand enhance its bioactivity with *in situ* synthetized glycolipids displaying several chain lengths. It was expected that bringing

endogenous surfactants to MH which intrinsically presents a versatile pool of bioactive compounds would bring a real difference in terms of bactericidal effect. This endogenous pool of compounds present in MH is mainly composed of methylglyoxal (MGO), bee defensin-1, a vast range of flavonoids and phenolics [295].

The approach used for this work was first reported by Pöhnlein *et al.* and corresponds to a strategy of a "2-in-1" system in which sugars are part of the solvent and part of the substrates as they participate to the enzymatic conversion (Pöhnlein *et al*., 2015; further studies reviewed in Pätzold *et al*., 2019). This methodology which was applied first to Deep Eutectic Solvents (DESs) was successfully transferred to honey and agave syrup with chain lengths of esters ranging from C8 to C16 [279]. In the GOH mixture it can be assumed that fructose and glucose laurate were produced and are responsible for the effect that will be discussed further in the next section. According to preliminary results the C16 esters enriched honey mixtures were not included in the present work but rather myristate (C14) was chosen alongside C8, C10 and C12 vinyl esters substrates for the lipase-catalyzed transesterification. The immobilized lipase used is the versatile Novozym 435® it was expected that the reaction using vinyl ester with longer chains, notably C14, would be catalyzed in MH as it is clearly shown on the TLC analysis. The enzyme showed great flexibility towards the range of substrates it can accept and the type of media in which it can work. MH possessing as well low water content and low water activity [296] it can reverse lipase action and form ester bonds the same way it was described in previous work [297].

However, one question remains concerning the effect of the process on compounds naturally present in MH. Hydrogen peroxide has been showed to be the main factor for bioactivity in standard honey. This last compound can be found in MH but the unique component mainly responsible for its activity is the MGO which originates from conversion of dihydroxyacetone present naturally in Manuka nectar flower [298]. Both of these components are known to be temperature sensible and studies have shown that storage of MH at 37°C leads to an apparent non-enzymatic increase of MGO upon several days [299]. The process employed here to enrich MH uses 50°C for 48h, so in theory an increase of MGO should be observed and therefore an increase in bioactivity. Withal, MHWE which serves as a negative control since no glycolipids are present in this condition shows no increase in bioactivity compared to pure MH even after heat treatment (**Figure 23**). Therefore, it can be affirmed that glycolipids are responsible for the observed increase in bioactivity. Still, composition in MGO and related components after process could be an additional way of investigation.

5.4.1. Role of the Glycolipids in Bioactivity Enhancement

Previous discussion made obvious that glycolipids in honey are playing a role in the bioactivity of the mixture. Nonetheless, susceptibility test, stress assay and antibacterial test were applied in order to get a deeper understanding on how this class of bio-surfactants acts inside the honey mixtures and in what measure they enhance the overall bioactivity of MH.

Susceptibility test showed clear evidence of MH and glycolipid mixtures to affect different types of microorganisms. Albeit evidence that glycolipid in honey brings bioactivity enhancement is not very clear from this first experiment. Bacteria and yeast tested exhibit clear sensitivity to honey mixtures at full concentration. The high osmolarity and unique MGO compound among other factors of MH [300] could explain this remarkable effect. Nevertheless, the test with *Candida bombicola* suggests that enzymatic glycolipid synthesis in

honey could bring another strong antimicrobial factor to this sugar-supersaturated solution. The different sensitivities displayed by those microbes guided us to investigate further GOH mixture which seemed to be the most efficient out of all glycolipid enriched mixtures. A clear tendency of Gram+ pathogens being more sensible to all honey mixtures was highlighted then compared to the other tested microorganisms and visibly no further bioactivity enhancement against Gram+ species was brought to MH throughout this experiment. Bacteria are known to be intolerant to high osmolarity, thus explaining the overall effect on non-yeast species with or without glycolipids. Interestingly the low susceptibility for *P. putida* could be explained by the polysaccharide slime capsule *Pseudomonas* species possess [301]. However, the yeast species and Gram– pathogens gave high sensibilities to sugar ester enriched honey. Wagh *et al* already reported higher sensitivity of Gram+ bacteria compared to Gram– when exposed to sugar esters [302]. We can postulate that similar effect observed in this work is due to Gram+ bacteria and yeasts only possessing a single lipid membrane while Gram– bacteria are equipped with an additional one. Therefore, honey enriched with glycolipids renders Gram– bacteria and yeasts more sensible to the high osmolarity of honey but make almost no difference in bioactivity against Gram+ ones.

Yeasts, such as *C. bombicola*, normally tolerate high osmolarity [303] but in our case we observed an increased effect as the chain length of the hydrophobic tail is shortened such as C8>C10>C12, suggesting a higher toxicity of acyl derivates possessing short alkyl chain and no effect with pure unmodified MH. Toxicity of C8 fatty acid was previously reported against *Saccharomyces cerevisiae* showing a general tendency of yeasts to be sensible to relatively short saturated acid chains as it causes damage to the membrane by inducing cell leakage [304]. Interestingly *Candida bombicola* is a yeast able to produce sophorolipids [305], which is made of a glucose-derived di-saccharide acetylated with C16 or C18 fatty acid tails. Thus, giving a possible insight on why honey mixtures provided with long chain vinyl fatty acid (e.g., C14 and C16) have very little effect against *Candida bombicola* compared to C8 sugar esters. It is also important to mention that *Candida* genus is known to produce plethora of lipases that are commercially available [306]. Then, another conjecture could be that a lipase from *C. bombicola* is responsible for the observed effects as glucose-octanoate represents a substrate releasing C8 free acid being fatal to the yeast, as no effect was observed with MHWE and decreasing effect with increased chain length.

Although in this study an apathogenic yeast was surveyed, *Candida* represents the genus of the most common cause of fungal infections with *C. albicans* as the most prominent species [307]. Alongside *C. glabatra*, *C. tropicalis*, *C. parapsilosis* and *C. krusei*, these five species represent 90% of infections cases among the *Candida* genus [308]. Therefore, follow-up studies might focus the potential of the GOH mixture as fungistatic in addition, since *C. albicans* is known to grow as biofilms on human tissue and implanted medical devices [309].

The stress assay combined with an antibacterial test allowed quantitative insight on possible mechanisms involved into the microorganisms´ inhibition induced by MH and glycolipid enriched MH mixtures. For this test, *E. coli* was chosen as a whole cell biosensor (RGB-S) because it can be expressed in this microorganism. Reading of OD_{600} during the 24h incubation of the biosensor gave clear confirmation that GOH showed quicker and stronger inhibition potential than any other pure or glycolipid enriched MH at only 1.6% (v/v) concentration. In addition to that, a remarkable 4-hour lag phase can be observed at beginning of the incubation time with the GOH condition. Only concentrations ranging from 0 to 1.6 %/ (v/v) in broth

dilution were tested because of phenolics contained in honey interfering with the readings, any measurement above these concentrations were inoperable. Mixture composed of octanoate esters of honey sugars revealed a 6-fold increase of the physiological stress and 2.5-fold increase of the cytotoxic stress compared to MH while having no impact on the microbes' genetic material. Three main clarifications may be evoked in attempt to explain these observations. First off sugars esters are known for their antibacterial activities [12] because of their ability to disrupt membranes [310,311]. Second off, it appeared within our experiments that shorter chain length of the sugar ester resulted in an increase of bioactivity. Indeed shorter alkyl chain results in higher CMC (Critical Micelle Concentration) which seems to explain a higher antibacterial potential [34]. The toxicity of glycolipids towards pathogens inside the honey mixtures appears to increase as the chain length decreases which appears logic as the latter can greatly modulate physicochemical properties of the sugar esters [312]. Third off, since the glycolipids seem to spare DNA damage inside the cell, it is less likely to induce mutations due to genotoxic stress thus making it a good candidate for a potential antibacterial treatment that would not induce antimicrobial resistance [313–315]. Finally, antibacterial test highlighted better efficiency of GOH at 20% (v/v) as no inhibition was observed for MH for this same concentration in agar plate assay. As a correlation to previous stress assay experiment it can be confirmed that glycolipids bring a supplementary factor to the bioactivity of honey.

Despite these results and affirmations, similar experiments with isolated glycolipids produced in MH should be carried in a further investigation to potentially highlight a synergy between honey and glycolipids. As honey and DESs are physico-chemically related, a synergy can be indeed suggested as DES formulations containing bioactive compounds exhibit such synergetic behavior [316–319]. Giving those facts we can hypothesize that glycolipid bioactivity is enhanced by honey and vice versa due to multiple physico-chemical factors inherent to both participants.

5.5. Conclusions

It was shown that production of glycolipids in MH, structurally identified as fructose and glucose laurate in previous work, catalyzed by the lipase formulation Novozym 435® enhances bioactivity of the mixture.

Stress and antibacterial assay revealed bioactivity strengthening even upon high dilution factor. The mixture dubbed GOH made from vinyl octanoate sugar esters induced 6-fold physiological stress increase and 2.5-fold cytotoxic stress increase compared to normal MH alongside a stronger and quicker inhibition based on the OD_{600} reading. On the other hand, no significant genotoxic stress of *E. coli* was measured. Such glycolipid enriched honey mixture represents potential tool to treat rapidly but also efficiently wounds that are susceptible of getting infected. In a practical case, they could be administered in a pasture-like form. Nonetheless, more investigation regarding the effect of isolated glycolipids produced herein should be carried out to highlight a suspected synergistic effect.

6. Final Conclusion and Outlook

The present dissertation dealt with glycolipids production which were presented as a class of ecological (bio)surfactants exhibiting excellent foaming abilities, wetting abilities and antimicrobial activity. This work highlighted initially the screening of 16 commercially available lipase formulations for the DES mediated and lipase-catalyzed production of glycolipid sorbitol-6-O-laurate in a "2-in-1" DES system. Doing so, we outlined the need for enlargement of immobilization materials and techniques for enzymes. Promising CLEAs revealed themselves not as performant and practical as their standard bead-adsorbed analogues, despite a higher mass of catalytic material. The structure of our model compound was elucidated and identified, with thorough spectroscopy and spectrometry analysis which should be a "must have" for all identification strategies of new surfactants. The influence of several factors, on our process, was determined such as time of reaction, enzyme dosage, substrate concentration and water content when using Novozym 435® as a biocatalyst owing to proper analytics. The conversion yield reached therein, corresponds to a 7-fold increase in performance from the original proof of concept of this system. In addition, evidence of the possible scalability of such process was brought. Using a stirred tank reactor (STR) allowed a batch production of the glycolipid in a heterogenous system but displayed a 2-fold decrease of performance from the initial tube-scale. Thus, it was shown that despite obvious limitations, such as principally mass transfer, DESs demonstrate factually their potential for mediating bioprocesses. More effort should, therefore, be put toward not only enzyme and reactor technologies but also toward optimization and scale-up methods.

Parallelly, those results were compared to the ones obtained when carrying the production of SL in greener organic solvent alternatives. 2M2B turned out to be more profitable in terms of production yields and reaction time when comparing to the various solvent systems. Moreover, using microwave irradiation we demonstrated the importance of innovative heating method for the production of glycolipids. However, this observation was greater for a system with low viscosity, as mass transfer potentially still limits the conversion in DES, even under dielectric microwave heating. We brought thus nuance to the intense build-up DESs are currently undergoing as media for biocatalysis in scientific literature albeit encouraging ways of improvement for this technology are at hand. The efficiency of our downstream processing was investigated. The latter allowed complete recovery of sorbitol laurate after 4 cycles of liquid-liquid extraction from the DES-containing aqueous mixtures. Continuous extraction technology could make sense in the strategy that we developed.

Thirdly, a proof of concept for the lipid extraction from oleaginous biomass and the subsequent glycolipids formation using lipase-catalyzed reaction with microwave-assisted processing in acidified DES was made. The energy input necessary for such fully integrated process was logically limited, as no prior cell-disruption method was needed. Moreover, a technological match was highlighted between microwaves and our "2-in-1" system. Thereby, we outlined that any combination of sugars and lipids is virtually possible to prepare tailor-made glycolipids as any other oleaginous biomasses could be considered, enabling a simplified and fast glycolipid synthesis using raw biomass. However, only a limited amount of glycolipid was obtained following this procedure, making optimization required. It is therefore important to investigate higher temperatures, different lipase formulations, and develop kinetic determination methods to allow an optimized scale-up of this newly proofed

process. It is foreseeable that more concrete applications will emerge concerning this specific topic. In addition, screening for potential bioactivity of the glycolipid- and lipid-containing DES mixtures would constitute an interesting follow-up study.

Finally, to demonstrate the potential of glycolipids mixtures as bactericidal agents, it was shown that production of glycolipids in medicinal Manuka Honey, structurally identified as fructose and glucose laurate, catalyzed by the lipase formulation Novozym 435®, enhances the overall bioactivity of the honey mixture. Stress and antibacterial assay revealed bioactivity strengthening even upon high dilution factor. The mixture dubbed GOH made from octanoate sugar esters induced a 6-fold physiological stress increase and a 2.5-fold cytotoxic stress increase compared to normal MH alongside a stronger and quicker inhibition based on the OD_{600} reading. On the other hand, no significant genotoxic stress of *E. coli* was measured. Such glycolipid enriched honey mixture represents a potential tool to treat rapidly and efficiently wounds that are susceptible of getting infected without inducing mutations that can cause future treatment resistance. In a practical case, these mixtures or more largely glycolipid containing DES mixtures could be administered "as is", in a pasture-like form removing thus the purification step. Nonetheless, more investigation regarding the effect of isolated glycolipids produced herein should be carried out to highlight a suspected synergistic effect.

DESs or DES-like mixtures demonstrate, through an ever-expanding literature, their genuine potential for mediating sustainable bioprocesses and reduce waste management strategies. Thus, further investigation and development are necessary to overcome hindrances intrinsic to this media. As a matter of fact, not only limitations were highlighted but also research axis for a further intensification of enzymatic processing in DES, as it could potentially open new horizons for industrial biocatalysis. Mechanochemistry and solvent free reactions are promising strategies to further enhance the performances of such process. Recyclability but also wild release of DES mixtures should also be seriously considered.

To condense the various chapters, it was demonstrated that using innovative technologies, approaches, substrates and media, one can produce bioactive mixtures or pure compounds using lipases as biocatalysts in optimized and mild conditions. Such harmless, inexpensive and straightforward processes can be intensified to reach superior yields and productivity, demonstrating their potential for application in sustainable and industrial biocatalysis.

List of all References

1. Mulligan, C.N. Environmental Applications for Biosurfactants. *Environmental Pollution* **2005**, *133*, 183–198, doi:10.1016/J.ENVPOL.2004.06.009.
2. Shahbaz, K.; Mjalli, F.S.; Hashim, M.A.; Alnashef, I.M. Prediction of Deep Eutectic Solvents Densities at Different Temperatures. *Thermochimica Acta* **2011**, *515*, 67–72, doi:10.1016/j.tca.2010.12.022.
3. Sen, R.; Sivapathasekaran, C. Origin, Properties, Production and Purification of Microbial Surfactants as Molecules with Immense Commercial Potential. *Tenside Surfactants Detergents* **2017**, *54*, 92–107.
4. Pritzkow, W. Die Tenside, Herausgegeben von K. Kosswig Und H. Stache München, Wien. Carl-Hanser-Verlag 1993, ISBN 3-446-16201-1, 510 Seiten, 253 Bilder, 78 Tabellen, Format 26 × 22,5 Cm, Gebunden, DM 178,00, ÖS 1389,–, SFR 177,–. *Journal für Praktische Chemie/Chemiker-Zeitung* **1994**, *336*, 473–473, doi:10.1002/prac.19943360518.
5. Makkar, R.S.; Rockne, K.J. Comparison of Synthetic Surfactants and Biosurfactants in Enhancing Biodegradation of Polycyclic Aromatic Hydrocarbons. *Environmental toxicology and chemistry* **2003**, *22*, 2280–92.
6. Ivanković, T.; Hrenović, J. Surfactants in the Environment. *Archives of Industrial Hygiene and Toxicology* **2010**, *61*, 95–110, doi:10.2478/10004-1254-61-2010-1943.
7. Lourith, N.; Kanlayavattanakul, M. Natural Surfactants Used in Cosmetics: Glycolipids. *Int J Cosmet Sci* **2009**, *31*, 255–261, doi:10.1111/j.1468-2494.2009.00493.x.
8. Silva, R.D.C.F.S.; Almeida, D.G.; Rufino, R.D.; Luna, J.M.; Santos, V.A.; Sarubbo, L.A. Applications of Biosurfactants in the Petroleum Industry and the Remediation of Oil Spills. *International Journal of Molecular Sciences* **2014**, *15*, 12523–12542, doi:10.3390/ijms150712523.
9. Ducret, A.; Giroux, A.; Trani, M.; Lortie, R. Characterization of Enzymatically Prepared Biosurfactants. *Journal of the American Oil Chemists' Society* **1996**, *73*, 109–113, doi:10.1007/BF02523456.
10. Tarahomjoo, S.; Alemzadeh, I. Surfactant Production by an Enzymatic Method. *Enzyme and Microbial Technology* **2003**, *33*, 33–37, doi:10.1016/S0141-0229(03)00085-1.
11. Recke, V.K.; Beyrle, C.; Gerlitzki, M.; Hausmann, R.; Syldatk, C.; Wray, V.; Tokuda, H.; Suzuki, N.; Lang, S. Lipase-Catalyzed Acylation of Microbial Mannosylerythritol Lipids (Biosurfactants) and Their Characterization. *Carbohydrate Research* **2013**, *373*, 82–88, doi:10.1016/j.carres.2013.03.013.
12. Altenbach, H.-J.; Ihizane, R.; Jakob, B.; Lange, K.; Schneider, M.; Yilmaz, Z.; Nandi, S. Synthesis and Characterization of Novel Surfactants: Combination Products of Fatty Acids, Hydroxycarboxylic Acids and Alcohols. *Journal of Surfactants and Detergents* **2010**, *13*, 399–407, doi:10.1007/s11743-010-1185-8.
13. Harada, S.; Yokomizo, K.; Monde, K.; Maeda, Y.; Yusa, K. A Broad Antiviral Neutral Glycolipid, Fattiviracin FV-8, Is a Membrane Fluidity Modulator. *Cellular Microbiology* **2007**, *9*, 196–203, doi:10.1111/j.1462-5822.2006.00781.x.
14. Teichmann, B.; Linne, U.; Hewald, S.; Marahiel, M.A.; Bölker, M. A Biosynthetic Gene Cluster for a Secreted Cellobiose Lipid with Antifungal Activity from Ustilago Maydis. *Molecular Microbiology* **2007**, *66*, 525–533, doi:10.1111/j.1365-2958.2007.05941.x.
15. Recke, V.K.; Gerlitzki, M.; Hausmann, R.; Syldatk, C.; Wray, V.; Tokuda, H.; Suzuki, N.; Lang, S. Enzymatic Production of Modified 2-Dodecyl-Sophorosides

(Biosurfactants) and Their Characterization. *European Journal of Lipid Science and Technology* **2013**, *115*, 452–463, doi:10.1002/ejlt.201300012.
16. Lang, S.; Trowitzsch-Kienast, W. *Biotenside*; Chemie in der Praxis; 1. Aufl.; Teubner: Stuttgart, 2002; ISBN 978-3-519-03615-9.
17. Müller, M.M.; Kügler, J.H.; Henkel, M.; Gerlitzki, M.; Hörmann, B.; Pöhnlein, M.; Syldatk, C.; Hausmann, R. Rhamnolipids-Next Generation Surfactants? *Journal of Biotechnology* **2012**, *162*, 366–380, doi:10.1016/j.jbiotec.2012.05.022.
18. Marchant, R.; Banat, I.M. Biosurfactants: A Sustainable Replacement for Chemical Surfactants? *Biotechnology Letters* **2012**, *34*, 1597–1605, doi:10.1007/s10529-012-0956-x.
19. Imura, T.; Ohta, N.; Inoue, K.; Yagi, N.; Negishi, H.; Yanagishita, H.; Kitamoto, D. Naturally Engineered Glycolipid Biosurfactants Leading to Distinctive Self-Assembled Structures. *Chemistry - A European Journal* **2006**, *12*, 2434–2440, doi:10.1002/chem.200501199.
20. Bolker, B.M.; Brooks, M.E.; Clark, C.J.; Geange, S.W.; Poulsen, J.R.; Stevens, M.H.H.; White, J.-S.S. Generalized Linear Mixed Models: A Practical Guide for Ecology and Evolution. *Trends in Ecology & Evolution* **2009**, *24*, 127–135.
21. Lin, J.T.; Riedel, S.; Kinne, R. The Use of Octyl Beta-D-Glucoside as Detergent for Hog Kidney Brush Border Membrane. *Biochimica et biophysica acta* **1979**, *557*, 179–87.
22. Svensson, D.; Ulvenlund, S.; Adlercreutz, P. Enzymatic Route to Alkyl Glycosides Having Oligomeric Head Groups. *Green Chemistry* **2009**, *11*, 1222–1226, doi:10.1039/B904849A.
23. Rybinski, W. von; Hill, K. Alkyl Polyglycosides—Properties and Applications of a New Class of Surfactants. *Angewandte Chemie International Edition* **1998**, *37*, 1328–1345, doi:10.1002/(SICI)1521-3773(19980605)37:10<1328::AID-ANIE1328>3.0.CO;2-9.
24. Izawa, S.; Sakai-Tomita, Y.; Kinomura, K.; Kitazawa, S.; Tsuda, M.; Tsuchiya, T. Introduction of a Series of Alkyl Thiomaltosides, Useful New Non-Ionic Detergents, to Membrane Biochemistry. *J. Biochem.* **1993**, *113*, 573–576, doi:10.1093/oxfordjournals.jbchem.a124085.
25. van Dongen, J.J.M.; Langerak, A.W.; Brüggemann, M.; Evans, P. a. S.; Hummel, M.; Lavender, F.L.; Delabesse, E.; Davi, F.; Schuuring, E.; García-Sanz, R.; et al. Design and Standardization of PCR Primers and Protocols for Detection of Clonal Immunoglobulin and T-Cell Receptor Gene Recombinations in Suspect Lymphoproliferations: Report of the BIOMED-2 Concerted Action BMH4-CT98-3936. *Leukemia* **2003**, *17*, 2257–2317, doi:10.1038/sj.leu.2403202.
26. Drouet, P.; Zhang, M.; Legoy, M.D. Enzymatic Synthesis of Alkyl P-D-Xylosides by Transxylosylation and Reverse Hydrolysis. **1994**, *43*, 1075–1080.
27. Milkereit, G.; Garamus, V.M.; Veermans, K.; Willumeit, R.; Vill, V. Structures of Micelles Formed by Synthetic Alkyl Glycosides with Unsaturated Alkyl Chains. *J Colloid Interface Sci* **2005**, *284*, 704–713, doi:10.1016/j.jcis.2004.10.039.
28. Banat, I.M.; Makkar, R.S.; Cameotra, S.S. Potential Commercial Applications of Microbial Surfactants. *Applied microbiology and biotechnology* **2000**, *53*, 495–508, doi:10.1007/s002530051648.
29. Igarashi, K. The Koenigs-Knorr Reaction. In; 1977; pp. 243–283.
30. Biermann, U.; Bornscheuer, U.; Meier, M.A.R.; Metzger, J.O.; Schäfer, H.J. Oils and Fats as Renewable Raw Materials in Chemistry. *Angew. Chem. Int. Ed.* **2011**, *50*, 3854–3871, doi:10.1002/anie.201002767.

31. Youssef, N.H.; Duncan, K.E.; McInerney, M.J. Importance of 3-Hydroxy Fatty Acid Composition of Lipopeptides for Biosurfactant Activity. *Applied and Environmental Microbiology* **2005**, *71*, 7690–7695, doi:10.1128/AEM.71.12.7690-7695.2005.
32. Bornscheuer, U.T. *Lipid Modification by Enzymes and Engineered Microbes*; Elsevier, 2018; ISBN 978-0-12-813168-8.
33. Stubenrauch, C. Sugar Surfactants — Aggregation, Interfacial, and Adsorption Phenomena. *Current Opinion in Colloid & Interface Science* **2001**, *6*, 160–170, doi:10.1016/S1359-0294(01)00080-2.
34. Razafindralambo, H.; Blecker, C.; Paquot, M. Carbohydrate-Based Surfactants: Structure-Activity Relationships. *Advances in Chemical Engineering* **2012**, doi:10.5772/34971.
35. Polat, T.; Bazin, H.G.; Linhardt, R.J. Enzyme Catalyzed Regioselective Synthesis of Sucrose Fatty Acid Ester Surfactants. *Journal of Carbohydrate Chemistry* **1997**, *16*, 1319–1325, doi:10.1080/07328309708005752.
36. Ducret, A.; Giroux, A.; Trani, M.; Lortie, R. Enzymatic Preparation of Biosurfactants from Sugars or Sugar Alcohols and Fatty Acids in Organic Media under Reduced Pressure. *Biotechnology and Bioengineering* **1995**, *48*, 214–221, doi:10.1002/bit.260480308.
37. Ferrer, M.; Comelles, F.; Plou, F.J.; Cruces, M.A.; Fuentes, G.; Parra, J.L.; Ballesteros, A. Comparative Surface Activities of Di- and Trisaccharide Fatty Acid Esters. *Langmuir* **2002**, *18*, 667–673, doi:10.1021/la010727g.
38. Husband, F.A.; Sarney, D.B.; Barnard, M.J.; Wilde, P.J. Comparison of Foaming and Interfacial Properties of Pure Sucrose Monolaurates, Dilaurate and Commercial Preparations. *Food Hydrocolloids* **1998**, *12*, 237–244, doi:10.1016/S0268-005X(98)00036-8.
39. Bhattacharya, S.; Acharya, S.N.G. Vesicle and Tubular Microstructure Formation from Synthetic Sugar-Linked Amphiphiles. Evidence of Vesicle Formation from Single-Chain Amphiphiles Bearing a Disaccharide Headgroup. *Langmuir* **2000**, *16*, 87–97, doi:10.1021/la990468j.
40. Razafindralambo, H.; Blecker, C.; Mezdour, S.; Deroanne, C.; Crowet, J.-M.; Brasseur, R.; Lins, L.; Paquot, M. Impacts of the Carbonyl Group Location of Ester Bond on Interfacial Properties of Sugar-Based Surfactants: Experimental and Computational Evidences. *J. Phys. Chem. B* **2009**, *113*, 8872–8877, doi:10.1021/jp903187f.
41. Fukui, S.; Tanaka, A. Enzymatic Reactions in Organic Solvents. *Endeavour* **1985**, *9*, 10–17, doi:10.1016/0160-9327(85)90004-3.
42. Zaks, A.; Russell, A.J. Enzymes in Organic Solvents: Properties and Applications. *Journal of Biotechnology* **1988**, *8*, 259–269, doi:10.1016/0168-1656(88)90018-1.
43. Zaks, A.; Klibanov, A.M. The Effect of Water on Enzyme Action in Organic Media. *Journal of Biological Chemistry* **1988**, *263*, 8017–8021.
44. Carrea, G.; Ottolina, G.; Riva, S. Role of Solvents in the Control of Enzyme Selectivity in Organic Media. *Trends in Biotechnology* **1995**, *13*, 63–70, doi:10.1016/S0167-7799(00)88907-6.
45. Chamouleau, F.; Coulon, D.; Girardin, M.; Ghoul, M. Influence of Water Activity and Water Content on Sugar Esters Lipase-Catalyzed Synthesis in Organic Media. In Proceedings of the Journal of Molecular Catalysis - B Enzymatic; January 2001; Vol. 11, pp. 949–954.
46. Pöhnlein, M.; Ulrich, J.; Kirschhöfer, F.; Nusser, M.; Muhle-Goll, C.; Kannengiesser, B.; Brenner-Weiß, G.; Luy, B.; Liese, A.; Syldatk, C.; et al. Lipase-Catalyzed Synthesis of Glucose-6-O-Hexanoate in Deep Eutectic Solvents. *European Journal of Lipid Science and Technology* **2015**, *117*, 161–166, doi:10.1002/ejlt.201400459.

47. Kumar, A.; Dhar, K.; Kanwar, S.S.; Arora, P.K. Lipase Catalysis in Organic Solvents: Advantages and Applications. *Biol Proced Online* **2016**, *18*, doi:10.1186/s12575-016-0033-2.
48. Shin, D.W.; Mai, N.L.; Bae, S.-W.; Koo, Y.-M. Enhanced Lipase-Catalyzed Synthesis of Sugar Fatty Acid Esters Using Supersaturated Sugar Solution in Ionic Liquids. *Enzyme and Microbial Technology* **2019**, *126*, 18–23, doi:10.1016/j.enzmictec.2019.03.004.
49. Qin, J.; Zou, X.; Lv, S.; Jin, Q.; Wang, X. Influence of Ionic Liquids on Lipase Activity and Stability in Alcoholysis Reactions. *RSC Advances* **2016**, *6*, 87703–87709, doi:10.1039/C6RA19181A.
50. Stamatis, H.; Sereti, V.; Kolisis, F.N. Lipase-Catalyzed Synthesis of Sugar Fatty Acid Esters in Supercritical Carbon Dioxide. In *Enzymes in Nonaqueous Solvents*; Humana Press: New Jersey, 2001; Vol. 15, pp. 517–522 ISBN 978-1-59259-112-1.
51. Chang, P.; Zhang, Z.; Tang, S. Lipase-Catalyzed Synthesis of Sugar Ester in Mixed Biphasic System of Ionic Liquids and Supercritical Carbon Dioxide. *Journal of the Chinese Chemical Society* **2018**, *65*, 452–458, doi:10.1002/jccs.201700278.
52. Xu, K.; Wang, Y.; Huang, Y.; Li, N.; Wen, Q. A Green Deep Eutectic Solvent-Based Aqueous Two-Phase System for Protein Extracting. *Analytica Chimica Acta* **2015**, *864*, 9–20, doi:10.1016/j.aca.2015.01.026.
53. Zhao, K.-H.; Cai, Y.-Z.; Lin, X.-S.; Xiong, J.; Halling, P.J.; Yang, Z. Enzymatic Synthesis of Glucose-Based Fatty Acid Esters in Bisolvent Systems Containing Ionic Liquids or Deep Eutectic Solvents. *Molecules* **2016**, *21*, doi:10.3390/molecules21101294.
54. Abbott, A.P.; Capper, G.; Davies, D.L.; Rasheed, R.K.; Tambyrajah, V. Novel Solvent Properties of Choline Chloride/Urea Mixtures. *Chemical Communications* **2002**, *99*, 70–71, doi:10.1039/b210714g.
55. Abbott, A.P.; Boothby, D.; Capper, G.; Davies, D.L.; Rasheed, R. Deep Eutectic Solvents Formed Between Choline Chloride and Carboxylic Acids. *J. Am. Chem. Soc* **2004**, *126*, 9142, doi:10.1021/ja048266j.
56. Durand, E.; Lecomte, J.; Baréa, B.; Piombo, G.; Dubreucq, E.; Villeneuve, P. Evaluation of Deep Eutectic Solvents as New Media For Candida Antarctica B Catalyzed Reactions. *Process Biochemistry* **2012**, *47*, 2081–2089, doi:10.1016/j.procbio.2012.07.027.
57. Tang, B.; Row, K.H. Recent Developments in Deep Eutectic Solvents in Chemical Sciences. *Monatshefte für Chemie - Chemical Monthly* **2013**, *144*, 1427–1454, doi:10.1007/s00706-013-1050-3.
58. Durand, E.; Lecomte, J.; Villeneuve, P. Deep Eutectic Solvents: Synthesis, Application, and Focus on Lipase-Catalyzed Reactions. *European Journal of Lipid Science and Technology* **2013**, *115*, 379–385, doi:10.1002/ejlt.201200416.
59. Thuy Pham, T.P.; Cho, C.-W.; Yun, Y.-S. Environmental Fate and Toxicity of Ionic Liquids: A Review. *Water Research* **2010**, *44*, 352–372, doi:10.1016/j.watres.2009.09.030.
60. Di Gioia, M.L.; Nardi, M.; Costanzo, P.; De Nino, A.; Maiuolo, L.; Oliverio, M.; Procopio, A. Biorenewable Deep Eutectic Solvent for Selective and Scalable Conversion of Furfural into Cyclopentenone Derivatives. *Molecules (Basel, Switzerland)* **2018**, *23*, doi:10.3390/molecules23081891.
61. Paiva, A.; Craveiro, R.; Aroso, I.; Martins, M.; Reis, R.L.; Duarte, A.R.C. Natural Deep Eutectic Solvents – Solvents for the 21st Century. *ACS Sustainable Chemistry & Engineering* **2014**, *2*, 1063–1071, doi:10.1021/sc500096j.

62. Ghanem, A.; Aboul-Enein, H.Y. Lipase-Mediated Chiral Resolution of Racemates in Organic Solvents. *Tetrahedron: Asymmetry* **2004**, *15*, 3331–3351, doi:10.1016/j.tetasy.2004.09.019.
63. Adlercreutz, P. Immobilisation and Application of Lipases in Organic Media. *Chemical Society Reviews* **2013**, *42*, 6406, doi:10.1039/c3cs35446f.
64. Seino, H.; Uchibori, T.; Nishitani, T.; Inamasu, S. Enzymatic Synthesis of Carbohydrate Esters of Fatty Acid (I) Esterification of Sucrose, Glucose, Fructose and Sorbitol. *Journal of the American Oil Chemists' Society* **1984**, *61*, 1761–1765, doi:10.1007/BF02582144.
65. Chopineau, J.; McCafferty, F.D.; Therisod, M.; Klibanov, A.M. Production of Biosurfactants from Sugar Alcohols and Vegetable Oils Catalyzed by Lipases in a Nonaqueous Medium. *Biotechnology and bioengineering* **1987**, *31*, 208–14, doi:10.1002/bit.260310305.
66. Therisod, M.; Klibanov, A.M. Facile Enzymatic Preparation of Monoacylated Sugars in Pyridine. *Journal of the American Chemical Society* **1986**, *108*, 5638–5640, doi:10.1021/ja00278a053.
67. Neta, N.S.; Teixeira, J.A.; Rodrigues, L.R. Sugar Ester Surfactants: Enzymatic Synthesis and Applications in Food Industry. *Critical Reviews in Food Science and Nutrition* **2015**, *55*, 595–610, doi:10.1080/10408398.2012.667461.
68. Jaeger, K.E.; Eggert, T. Lipases for Biotechnology. *Current Opinion in Biotechnology* **2002**, *13*, 390–397, doi:10.1016/S0958-1669(02)00341-5.
69. Sheldon, R.A. Enzyme Immobilization: The Quest for Optimum Performance. *Advanced Synthesis & Catalysis* **2007**, *349*, 1289–1307, doi:10.1002/adsc.200700082.
70. Guajardo, N.; Ahumada, K.; Domínguez de María, P.; Schrebler, R.A. Remarkable Stability of Candida Antarctica Lipase B Immobilized via Cross-Linking Aggregates (CLEA) in Deep Eutectic Solvents. *Biocatalysis and Biotransformation* **2018**, 1–9, doi:10.1080/10242422.2018.1492567.
71. Oguntimein, G.B.; Erdmann, H.; Schmid, R.D. Lipase Catalysed Synthesis of Sugar Ester in Organic Solvents. *Biotechnology Letters* **1993**, *15*, 175–180, doi:10.1007/BF00133019.
72. Khaled, N.; Montet, D.; Pina, M.; Graille, J. Fructose Oleate Synthesis in a Fixed Catalyst Bed Reactor. *Biotechnology Letters* **1991**, *13*, 167–172, doi:10.1007/BF01025812.
73. Šabeder, S.; Habulin, M.; Knez, Ž. Lipase-Catalyzed Synthesis of Fatty Acid Fructose Esters. *Journal of Food Engineering* **2006**, *77*, 880–886, doi:10.1016/J.JFOODENG.2005.08.016.
74. Zaidan, U.H.; Abdul Rahman, M.B.; Othman, S.S.; Basri, M.; Abdulmalek, E.; Abdul Rahman, R.N.Z.R.; Salleh, A.B. Biocatalytic Production of Lactose Ester Catalysed by Mica-Based Immobilised Lipase. *Food Chemistry* **2012**, *131*, 199–205, doi:10.1016/j.foodchem.2011.08.060.
75. Pöhnlein, M.; Ulrich, J.; Kirschhöfer, F.; Nusser, M.; Muhle-Goll, C.; Kannengiesser, B.; Brenner-Weiß, G.; Luy, B.; Liese, A.; Syldatk, C.; et al. Lipase-Catalyzed Synthesis of Glucose-6- O -Hexanoate in Deep Eutectic Solvents. *European Journal of Lipid Science and Technology* **2015**, *117*, 161–166, doi:10.1002/ejlt.201400459.
76. Sakaki, K.; Aoyama, A.; Nakane, T.; Ikegami, T.; Negishi, H.; Watanabe, K.; Yanagishita, H. Enzymatic Synthesis of Sugar Esters in Organic Solvent Coupled with Pervaporation. *Desalination* **2006**, *193*, 260–266, doi:10.1016/j.desal.2005.06.063.
77. Habulin, M.; Šabeder, S.; Knez, Ž. Enzymatic Synthesis of Sugar Fatty Acid Esters in Organic Solvent and in Supercritical Carbon Dioxide and Their Antimicrobial Activity.

The Journal of Supercritical Fluids **2008**, *45*, 338–345, doi:10.1016/j.supflu.2008.01.002.
78. Siebenhaller, S.; Muhle-Goll, C.; Luy, B.; Kirschhöfer, F.; Brenner-Weiss, G.; Hiller, E.; Günther, M.; Rupp, S.; Zibek, S.; Syldatk, C. Sustainable Enzymatic Synthesis of Glycolipids in a Deep Eutectic Solvent System. *Journal of Molecular Catalysis B: Enzymatic* **2017**, 1–7, doi:10.1016/j.molcatb.2017.01.015.
79. Siebenhaller, S.; Hajek, T.; Muhle-Goll, C.; Himmelsbach, M.; Luy, B.; Kirschhöfer, F.; Brenner-Weiß, G.; Hahn, T.; Zibek, S.; Syldatk, C. Beechwood Carbohydrates for Enzymatic Synthesis of Sustainable Glycolipids. *Bioresources and Bioprocessing* **2017**, *4*, 25, doi:10.1186/s40643-017-0155-7.
80. Lee, S.H.; Ha, S.H.; Hiep, N.M.; Chang, W.-J.; Koo, Y.-M. Lipase-Catalyzed Synthesis of Glucose Fatty Acid Ester Using Ionic Liquids Mixtures. *Journal of Biotechnology* **2008**, *133*, 486–489, doi:10.1016/j.jbiotec.2007.11.001.
81. Abdulmalek, E.; Hamidon, N.F.; Abdul Rahman, M.B. Optimization and Characterization of Lipase Catalysed Synthesis of Xylose Caproate Ester in Organic Solvents. *Journal of Molecular Catalysis B: Enzymatic* **2016**, *132*, 1–4, doi:10.1016/j.molcatb.2016.06.010.
82. Cauglia, F.; Canepa, P. The Enzymatic Synthesis of Glucosylmyristate as a Reaction Model for General Considerations on "sugar Esters" Production. *Bioresource Technology* 2008, *99*, 4065–4072.
83. Šabeder, S.; Habulin, M.; Knez, Ž. Lipase-Catalyzed Synthesis of Fatty Acid Fructose Esters. *Journal of Food Engineering* **2006**, *77*, 880–886, doi:10.1016/j.jfoodeng.2005.08.016.
84. Smith*, E.L. Deep Eutectic Solvents (DESs) and the Metal Finishing Industry: Where Are They Now? *Transactions of the IMF* **2013**, *91*, 241–248, doi:10.1179/0020296713Z.000000000120.
85. Crawford, D.E.; Wright, L.A.; James, S.L.; Abbott, A.P. Efficient Continuous Synthesis of High Purity Deep Eutectic Solvents by Twin Screw Extrusion. *Chemical Communications* **2016**, *52*, 4215–4218, doi:10.1039/C5CC09685E.
86. Siebenhaller, S.; Kirchhoff, J.; Kirschhöfer, F.; Brenner-Weiß, G.; Muhle-Goll, C.; Luy, B.; Haitz, F.; Hahn, T.; Zibek, S.; Syldatk, C.; et al. Integrated Process for the Enzymatic Production of Fatty Acid Sugar Esters Completely Based on Lignocellulosic Substrates. *Frontiers in Chemistry* **2018**, *6*, doi:10.3389/fchem.2018.00421.
87. van Rantwijk, F.; Woudenberg-van Oosterom, M.; Sheldon, R.A. Glycosidase-Catalysed Synthesis of Alkyl Glycosides. *Journal of Molecular Catalysis B: Enzymatic* **1999**, *6*, 511–532, doi:10.1016/S1381-1177(99)00042-9.
88. Crout, D.H.; Vic, G. Glycosidases and Glycosyl Transferases in Glycoside and Oligosaccharide Synthesis. *Curr Opin Chem Biol* **1998**, *2*, 98–111.
89. Wong, C.-H. Enzymatic and Chemo-Enzymatic Synthesis of Carbohydrates. *Pure and Applied Chemistry* **1995**, *67*, 1609–1616, doi:10.1351/pac199567101609.
90. Fernández-Mayoralas, A. Synthesis and modification of carbohydrates using glycosidases and lipases. In *Glycoscience Synthesis of Oligosaccharides and Glycoconjugates*; Driguez, H., Thiem, J., Eds.; Topics in Current Chemistry; Springer Berlin Heidelberg: Berlin, Heidelberg, 1997; pp. 1–20 ISBN 978-3-540-49629-8.
91. Vic, G.; Thomas, D.; Crout, D.H.G. Solvent Effect on Enzyme-Catalyzed Synthesis of ??-D-Glucosides Using the Reverse Hydrolysis Method: Application to the Preparative-Scale Synthesis of 2-Hydroxybenzyl and Octyl ??-D-Glucopyranosides. *Enzyme and Microbial Technology* **1997**, *20*, 597–603, doi:10.1016/S0141-0229(96)00201-3.
92. Millqvist-Fureby, A.; Gill, I.S.; Vulfson, E.N. Enzymatic Transformations in Supersaturated Substrate Solutions: I. A General Study with Glycosidases.

Biotechnology and Bioengineering **1998**, *60*, 190–196, doi:10.1002/(SICI)1097-0290(19981020)60:2<190::AID-BIT6>3.0.CO;2-I.
93. Ismail, A.; Soultani, S.; Ghoul, M. Enzymatic-Catalyzed Synthesis of Alkylglycosides in Monophasic and Biphasic Systems. I. The Transglycosylation Reaction. *Journal of Biotechnology* **1999**, *69*, 135–143, doi:10.1016/S0168-1656(99)00041-3.
94. Kouptsova, O.S.; Klyachko, N.L.; Levashov, A. V. Synthesis of Alkyl Glycosides Catalyzed by SS-Glycosidases in a System of Reverse Micelles. *Russian Journal of Bioorganic Chemistry* **2001**, *27*, 380–384, doi:10.1023/A:1012936702457.
95. Gumel, A.M.; Annuar, M.S.M.; Heidelberg, T.; Chisti, Y. Lipase Mediated Synthesis of Sugar Fatty Acid Esters. *Process Biochemistry* **2011**, *46*, 2079–2090, doi:10.1016/j.procbio.2011.07.021.
96. Pleiss, J.; Fischer, M.; Schmid, R.D. Anatomy of Lipase Binding Sites: The Scissile Fatty Acid Binding Site. *Chemistry and Physics of Lipids* **1998**, *93*, 67–80, doi:10.1016/S0009-3084(98)00030-9.
97. Millqvist-Fureby, A.; Gao, C.; Vulfson, E.N. Regioselective Synthesis of Ethoxylated Glycoside Esters Using □-Glucosidase in Supersaturated Solutions and Lipases in Organic Solvents. *BIOTECHNOLOGY AND BIOENGINEERING* **1998**, *59*, 7.
98. Otto, R.T.; Bornscheuer, U.T.; Syldatk, C.; Schmid, R.D. Synthesis of Aromatic N-Alkyl-Glucoside Esters in a Coupled β-Glucosidase and Lipase Reaction. *Biotechnology Letters* **1998**, *20*, 437–440, doi:10.1023/A:1005367215303.
99. Panintrarux, C.; Adachi, S.; Araki, Y.; Kimura, Y.; Matsuno, R. Equilibrium Yield of N-Alkyl-β-d-Glucoside through Condensation of Glucose and n-Alcohol by β-Glucosidase in a Biphasic System. *Enzyme and Microbial Technology* **1995**, *17*, 32–40, doi:10.1016/0141-0229(94)00082-3.
100. Yi, Q.; Sarney, D.B.; Khan, J.A.; Vulfson, E.N. Microencapsulated □-Glucosidase. *BIOTECHNOLOGY AND BIOENGINEERING* **1998**, *60*, 6.
101. Franzetti, A.; Gandolfi, I.; Bestetti, G.; Smyth, T.J.P.; Banat, I.M. Production and Applications of Trehalose Lipid Biosurfactants. *European Journal of Lipid Science and Technology* **2010**, *112*, 617–627, doi:10.1002/ejlt.200900162.
102. *Biosurfactants: From Genes to Applications*; Soberón-Chávez, G., Ed.; Microbiology Monographs; Springer-Verlag: Berlin Heidelberg, 2011; ISBN 978-3-642-14489-9.
103. Gudiña, E.J.; Rangarajan, V.; Sen, R.; Rodrigues, L.R. Potential Therapeutic Applications of Biosurfactants. *Trends Pharmacol. Sci.* **2013**, *34*, 667–675, doi:10.1016/j.tips.2013.10.002.
104. Sudo, T.; Zhao, X.; Wakamatsu, Y.; Shibahara, M.; Nomura, N.; Nakahara, T.; Suzuki, A.; Kobayashi, Y.; Jin, C.; Murata, T.; et al. Induction of the Differentiation of Human HL-60 Promyelocytic Leukemia Cell Line by Succinoyl Trehalose Lipids. *Cytotechnology* **2000**, *33*, 259–264, doi:10.1023/A:1008137817944.
105. Zaragoza, A.; Aranda, F.J.; Espuny, M.J.; Teruel, J.A.; Marqués, A.; Manresa, A.; Ortiz, A. Mechanism of Membrane Permeabilization by a Bacterial Trehalose Lipid Biosurfactant Produced by Rhodococcus Sp. *Langmuir* **2009**, *25*, 7892–7898, doi:10.1021/la900480q.
106. Adlercreutz, D.; Tufvesson, P.; Karlsson, A.; Hatti-Kaul, R. ORIGINAL RESEARCH: Alkanolamide Biosurfactants: Techno-Economic Evaluation of Biocatalytic versus Chemical Production. *Industrial Biotechnology* **2010**, *6*, 204–211, doi:10.1089/ind.2010.6.204.
107. Kosaric, N.; Sukan, F.V.; Sukan, F.V. *Biosurfactants : Production and Utilization-Processes, Technologies, and Economics*; CRC Press, 2014; ISBN 978-0-429-16956-4.
108. Queneau, Y.; Chambert, S.; Besset, C.; Cheaib, R. Recent Progress in the Synthesis of Carbohydrate-Based Amphiphilic Materials: The Examples of Sucrose and

Isomaltulose. *Carbohydrate Research* **2008**, *343*, 1999–2009, doi:10.1016/j.carres.2008.02.008.
109. Rau, A.H.; Speckman, D.T. Process of Preparing Alkylsaccharides 1984.
110. Paulino, B.N.; Pessôa, M.G.; Mano, M.C.R.; Molina, G.; Neri-Numa, I.A.; Pastore, G.M. Current Status in Biotechnological Production and Applications of Glycolipid Biosurfactants. *Appl Microbiol Biotechnol* **2016**, *100*, 10265–10293, doi:10.1007/s00253-016-7980-z.
111. Inès, M.; Dhouha, G. Glycolipid Biosurfactants: Potential Related Biomedical and Biotechnological Applications. *Carbohydr Res* **2015**, *416*, 59–69, doi:10.1016/j.carres.2015.07.016.
112. Dhasayan, A.; Kiran, G.S.; Selvin, J. Production and Characterisation of Glycolipid Biosurfactant by Halomonas Sp. MB-30 for Potential Application in Enhanced Oil Recovery. *Appl Biochem Biotechnol* **2014**, *174*, 2571–2584, doi:10.1007/s12010-014-1209-3.
113. Selmair, P.L.; Koehler, P. Role of Glycolipids in Breadmaking. *Lipid Technology* **2010**, *22*, 7–10, doi:https://doi.org/10.1002/lite.200900069.
114. Banat, I.M.; Carboué, Q.; Saucedo-Castañeda, G.; de Jesús Cázares-Marinero, J. Biosurfactants: The Green Generation of Speciality Chemicals and Potential Production Using Solid-State Fermentation (SSF) Technology. *Bioresource Technology* **2021**, *320*, 124222, doi:10.1016/j.biortech.2020.124222.
115. Anastas, P.T. Benign by Design Chemistry. In *Benign by Design*; Anastas, P.T., Farris, C.A., Eds.; American Chemical Society: Washington, DC, 1994; Vol. 577, pp. 2–22 ISBN 978-0-8412-3053-8.
116. Anastas, P.; Warner, J. *Green Chemistry: Theory and Practice*; Oxford University Press: Oxford, New York, 2000; ISBN 978-0-19-850698-0.
117. Grüninger, J.; Delavault, A.; Ochsenreither, K. Enzymatic Glycolipid Surfactant Synthesis from Renewables. *Process Biochemistry* **2019**, *87*, 45–54, doi:10.1016/j.procbio.2019.09.023.
118. Lipshutz, B.H.; Ghorai, S. Transitioning Organic Synthesis from Organic Solvents to Water. What's Your E Factor? *Green Chem.* **2014**, *16*, 3660–3679, doi:10.1039/C4GC00503A.
119. Pöhnlein, M.; Ulrich, J.; Kirschhöfer, F.; Nusser, M.; Muhle-Goll, C.; Kannengiesser, B.; Brenner-Weiß, G.; Luy, B.; Liese, A.; Syldatk, C.; et al. Lipase-Catalyzed Synthesis of Glucose-6-O-Hexanoate in Deep Eutectic Solvents. *European Journal of Lipid Science and Technology* **2015**, *117*, 161–166, doi:10.1002/ejlt.201400459.
120. Abbott, A.P.; Capper, G.; Davies, D.L.; Rasheed, R.K.; Tambyrajah, V. Novel Solvent Properties of Choline Chloride/Urea MixturesElectronic Supplementary Information (ESI) Available: Spectroscopic Data. See Http://Www.Rsc.Org/Suppdata/Cc/B2/B210714g/. *Chemical Communications* **2003**, 70–71, doi:10.1039/b210714g.
121. Durand, E.; Lecomte, J.; Villeneuve, P. From Green Chemistry to Nature: The Versatile Role of Low Transition Temperature Mixtures. *Biochimie* **2016**, *120*, 119–123, doi:10.1016/j.biochi.2015.09.019.
122. Di Gioia, M.L.; Nardi, M.; Costanzo, P.; De Nino, A.; Maiuolo, L.; Oliverio, M.; Procopio, A. Biorenewable Deep Eutectic Solvent for Selective and Scalable Conversion of Furfural into Cyclopentenone Derivatives. *Molecules* **2018**, *23*, doi:10.3390/molecules23081891.
123. Piemontese, L.; Sergio, R.; Rinaldo, F.; Brunetti, L.; Perna, F.M.; Santos, M.A.; Capriati, V. Deep Eutectic Solvents as Effective Reaction Media for the Synthesis of 2-Hydroxyphenylbenzimidazole-Based Scaffolds En Route to Donepezil-Like Compounds. *Molecules* **2020**, *25*, 574, doi:10.3390/molecules25030574.

124. Di Gioia, M.L.; Cassano, R.; Costanzo, P.; Herrera Cano, N.; Maiuolo, L.; Nardi, M.; Nicoletta, F.P.; Oliverio, M.; Procopio, A. Green Synthesis of Privileged Benzimidazole Scaffolds Using Active Deep Eutectic Solvent. *Molecules* **2019**, *24*, 2885, doi:10.3390/molecules24162885.
125. Bonacci, S.; Di Gioia, M.L.; Costanzo, P.; Maiuolo, L.; Tallarico, S.; Nardi, M. Natural Deep Eutectic Solvent as Extraction Media for the Main Phenolic Compounds from Olive Oil Processing Wastes. *Antioxidants* **2020**, *9*, 513, doi:10.3390/antiox9060513.
126. Pätzold, M.; Siebenhaller, S.; Kara, S.; Liese, A.; Syldatk, C.; Holtmann, D. Deep Eutectic Solvents as Efficient Solvents in Biocatalysis. *Trends in Biotechnology* **2019**, *37*, 943–959, doi:10.1016/j.tibtech.2019.03.007.
127. Siebenhaller, S.; Muhle-Goll, C.; Luy, B.; Kirschhöfer, F.; Brenner-Weiss, G.; Hiller, E.; Günther, M.; Rupp, S.; Zibek, S.; Syldatk, C. Sustainable Enzymatic Synthesis of Glycolipids in a Deep Eutectic Solvent System. *Journal of Molecular Catalysis B: Enzymatic* **2016**, *133*, S281–S287, doi:10.1016/j.molcatb.2017.01.015.
128. Bornscheuer, U.T.; Yamane, T. Fatty Acid Vinyl Esters as Acylating Agents: A New Method for the Enzymatic Synthesis of Monoacylglycerols. *J Am Oil Chem Soc* **1995**, *72*, 193–197, doi:10.1007/BF02638899.
129. Jeon, G.-J.; Park, O.-J.; Hur, B.-K.; Yang, J.-W. Enzymatic Synthesis of Amino Acid-Sugar Alcohol Conjugates in Organic Media. *Biotechnology Letters* **2001**, *23*, 929–934, doi:10.1023/A:1010598031929.
130. Jahangiri, A.; Møller, A.H.; Danielsen, M.; Madsen, B.; Joernsgaard, B.; Vaerbak, S.; Adlercreutz, P.; Dalsgaard, T.K. Hydrophilization of Bixin by Lipase-Catalyzed Transesterification with Sorbitol. *Food Chemistry* **2018**, *268*, 203–209, doi:10.1016/j.foodchem.2018.06.085.
131. Kim, H.J.; Youn, S.H.; Shin, C.S. Lipase-Catalyzed Synthesis of Sorbitol–Fatty Acid Esters at Extremely High Substrate Concentrations. *Journal of Biotechnology* **2006**, *123*, 174–184, doi:10.1016/j.jbiotec.2005.11.006.
132. Seino, H.; Uchibori, T.; Nishitani, T.; Inamasu, S. Enzymatic Synthesis of Carbohydrate Esters of Fatty Acid (I) Esterification of Sucrose, Glucose, Fructose and Sorbitol. *Journal of the American Oil Chemists' Society* **1984**, *61*, 1761–1765, doi:10.1007/BF02582144.
133. Delavault, A.; Ochs, K.; Gorte, O.; Syldatk, C.; Durand, E.; Ochsenreither, K. Microwave-Assisted One-Pot Lipid Extraction and Glycolipid Production from Oleaginous Yeast Saitozyma Podzolica in Sugar Alcohol-Based Media. **2021**, 16.
134. Elmowafy, E.; El-Derany, M.O.; Biondo, F.; Tiboni, M.; Casettari, L.; Soliman, M.E. Quercetin Loaded Monolaurate Sugar Esters-Based Niosomes: Sustained Release and Mutual Antioxidant—Hepatoprotective Interplay. *Pharmaceutics* **2020**, *12*, 143, doi:10.3390/pharmaceutics12020143.
135. Siebenhaller, S.; Kirchhoff, J.; Kirschhöfer, F.; Brenner-Weiß, G.; Muhle-Goll, C.; Luy, B.; Haitz, F.; Hahn, T.; Zibek, S.; Syldatk, C.; et al. Integrated Process for the Enzymatic Production of Fatty Acid Sugar Esters Completely Based on Lignocellulosic Substrates. *Front. Chem.* **2018**, *6*, doi:10.3389/fchem.2018.00421.
136. Hollenbach, R.; Ochsenreither, K.; Syldatk, C. Enzymatic Synthesis of Glucose Monodecanoate in a Hydrophobic Deep Eutectic Solvent. *International Journal of Molecular Sciences* **2020**, *21*, 4342, doi:10.3390/ijms21124342.
137. Dai, Y.; Witkamp, G.-J.; Verpoorte, R.; Choi, Y.H. Tailoring Properties of Natural Deep Eutectic Solvents with Water to Facilitate Their Applications. *Food Chemistry* **2015**, *187*, 14–19, doi:10.1016/j.foodchem.2015.03.123.
138. Hayyan, A.; Mjalli, F.S.; Alnashef, I.M.; Al-Wahaibi, Y.M.; Al-Wahaibi, T.; Hashim, M.A. Glucose-Based Deep Eutectic Solvents: Physical Properties. *Journal of Molecular Liquids* **2013**, *178*, 137–141, doi:10.1016/j.molliq.2012.11.025.

139. Hollenbach, R.; Bindereif, B.; van der Schaaf, U.S.; Ochsenreither, K.; Syldatk, C. Optimization of Glycolipid Synthesis in Hydrophilic Deep Eutectic Solvents. *Front. Bioeng. Biotechnol.* **2020**, *8*, 382, doi:10.3389/fbioe.2020.00382.
140. Chandra, P.; Enespa; Singh, R.; Arora, P.K. Microbial Lipases and Their Industrial Applications: A Comprehensive Review. *Microbial Cell Factories* **2020**, *19*, 169, doi:10.1186/s12934-020-01428-8.
141. Adlercreutz, P. Immobilisation and Application of Lipases in Organic Media. *Chemical Society Reviews* **2013**, *42*, 6406, doi:10.1039/c3cs35446f.
142. Arroyo, M.; Sánchez-Montero, J.M.; Sinisterra, J.V. Thermal Stabilization of Immobilized Lipase B from Candida Antarctica on Different Supports: Effect of Water Activity on Enzymatic Activity in Organic Media. *Enzyme and Microbial Technology* **1999**, *24*, 3–12, doi:10.1016/S0141-0229(98)00067-2.
143. Krasňan, V.; Stloukal, R.; Rosenberg, M.; Rebroš, M. Immobilization of Cells and Enzymes to LentiKats®. *Applied Microbiology and Biotechnology* **2016**, *100*, 2535–2553, doi:10.1007/s00253-016-7283-4.
144. Wahab, R.A.; Elias, N.; Abdullah, F.; Ghoshal, S.K. On the Taught New Tricks of Enzymes Immobilization: An All-Inclusive Overview. *Reactive and Functional Polymers* **2020**, *152*, 104613, doi:10.1016/j.reactfunctpolym.2020.104613.
145. Ortiz, C.; Ferreira, M.L.; Barbosa, O.; Santos, J.C.S. dos; Rodrigues, R.C.; Berenguer-Murcia, Á.; Briand, L.E.; Fernandez-Lafuente, R. Novozym 435: The "Perfect" Lipase Immobilized Biocatalyst? *Catal. Sci. Technol.* **2019**, *9*, 2380–2420, doi:10.1039/C9CY00415G.
146. Arana-Peña, S.; Lokha, Y.; Fernández-Lafuente, R. Immobilization of Eversa Lipase on Octyl Agarose Beads and Preliminary Characterization of Stability and Activity Features. *Catalysts* **2018**, *8*, 511, doi:10.3390/catal8110511.
147. Arana-Peña, S.; Lokha, Y.; Fernández-Lafuente, R. Immobilization on Octyl-Agarose Beads and Some Catalytic Features of Commercial Preparations of Lipase a from Candida Antarctica (Novocor ADL): Comparison with Immobilized Lipase B from Candida Antarctica. *Biotechnol Prog* **2019**, *35*, e2735, doi:10.1002/btpr.2735.
148. Souza, S.P. de; Almeida, R.A. de; Garcia, G.G.; Leão, R.A.; Bassut, J.; Souza, R.O. de; Itabaiana, I. Immobilization of Lipase B from Candida Antarctica on Epoxy-functionalized Silica: Characterization and Improving Biocatalytic Parameters. *Journal of Chemical Technology & Biotechnology* **2018**, *93*, 105–111, doi:10.1002/jctb.5327.
149. Mateo, C.; Palomo, J.M.; Fernandez-Lorente, G.; Guisan, J.M.; Fernandez-Lafuente, R. Improvement of Enzyme Activity, Stability and Selectivity via Immobilization Techniques. *Enzyme and Microbial Technology* **2007**, *40*, 1451–1463, doi:10.1016/j.enzmictec.2007.01.018.
150. Sheldon, R.A. Enzyme Immobilization: The Quest for Optimum Performance. *Advanced Synthesis & Catalysis* **2007**, *349*, 1289–1307, doi:10.1002/adsc.200700082.
151. Barbosa, O.; Ortiz, C.; Berenguer-Murcia, Á.; Torres, R.; Rodrigues, R.C.; Fernandez-Lafuente, R. Strategies for the One-Step Immobilization–Purification of Enzymes as Industrial Biocatalysts. *Biotechnology Advances* **2015**, *33*, 435–456, doi:10.1016/j.biotechadv.2015.03.006.
152. Rodrigues, R.C.; Ortiz, C.; Berenguer-Murcia, Á.; Torres, R.; Fernández-Lafuente, R. Modifying Enzyme Activity and Selectivity by Immobilization. *Chem Soc Rev* **2013**, *42*, 6290–6307, doi:10.1039/c2cs35231a.
153. Garcia-Galan, C.; Berenguer-Murcia, Á.; Fernandez-Lafuente, R.; Rodrigues, R.C. Potential of Different Enzyme Immobilization Strategies to Improve Enzyme Performance. *Advanced Synthesis & Catalysis* **2011**, *353*, 2885–2904, doi:https://doi.org/10.1002/adsc.201100534.

154. Brady, D.; Jordaan, J. Advances in Enzyme Immobilisation. *Biotechnol Lett* **2009**, *31*, 1639, doi:10.1007/s10529-009-0076-4.
155. Tran, D.N.; Balkus, K.J. Perspective of Recent Progress in Immobilization of Enzymes. *ACS Catal.* **2011**, *1*, 956–968, doi:10.1021/cs200124a.
156. Mohamad, N.R.; Marzuki, N.H.C.; Buang, N.A.; Huyop, F.; Wahab, R.A. An Overview of Technologies for Immobilization of Enzymes and Surface Analysis Techniques for Immobilized Enzymes. *Biotechnology & Biotechnological Equipment* **2015**, *29*, 205–220, doi:10.1080/13102818.2015.1008192.
157. Guzik, U.; Hupert-Kocurek, K.; Wojcieszyńska, D. Immobilization as a Strategy for Improving Enzyme Properties-Application to Oxidoreductases. *Molecules* **2014**, *19*, 8995–9018, doi:10.3390/molecules19078995.
158. Homaei, A.A.; Sariri, R.; Vianello, F.; Stevanato, R. Enzyme Immobilization: An Update. *J Chem Biol* **2013**, *6*, 185–205, doi:10.1007/s12154-013-0102-9.
159. Singh, R.K.; Tiwari, M.K.; Singh, R.; Lee, J.-K. From Protein Engineering to Immobilization: Promising Strategies for the Upgrade of Industrial Enzymes. *Int J Mol Sci* **2013**, *14*, 1232–1277, doi:10.3390/ijms14011232.
160. Liu, W.; Duan, F. Lipase-Catalyzed Transesterification of Epoxidized Soybean Oil to Prepare Epoxy Methyl Esters. *grasasaceites* **2018**, *69*, e247–e247, doi:10.3989/gya.1103172.
161. Sheldon, R.A. CHAPTER 14:Cross-linked Enzyme Aggregates (CLEAs): From Concept to Industrial Biocatalyst. In *Biocatalysis*; 2017; pp. 363–396.
162. Sheldon, R.A.; Schoevaart, R.; Van Langen, L.M. Cross-Linked Enzyme Aggregates (CLEAs): A Novel and Versatile Method for Enzyme Immobilization (a Review). *Biocatalysis and Biotransformation* **2005**, *23*, 141–147, doi:10.1080/10242420500183378.
163. Jegan Roy, J.; Emilia Abraham, T. Strategies in Making Cross-Linked Enzyme Crystals. *Chemical Reviews* **2004**, *104*, 3705–3722, doi:10.1021/cr0204707.
164. Sheldon, R.A.; Sorgedrager, M.; Janssen, M.H. Use of Cross-Linked Enzyme Aggregates (CLEAs) for Performing Biotransformations. *Chimica oggi* **2007**, *25*, 62.
165. Palomo, J.M.; Fuentes, M.; Fernández-Lorente, G.; Mateo, C.; Guisan, J.M.; Fernández-Lafuente, R. General Trend of Lipase to Self-Assemble Giving Bimolecular Aggregates Greatly Modifies the Enzyme Functionality. *Biomacromolecules* **2003**, *4*, 1–6, doi:10.1021/bm025729+.
166. Arutchelvi, J.I.; Bhaduri, S.; Uppara, P.V.; Doble, M. Mannosylerythritol Lipids: A Review. *Journal of Industrial Microbiology and Biotechnology* **2008**, *35*, 1559–1570, doi:10.1007/s10295-008-0460-4.
167. Saika, A.; Koike, H.; Fukuoka, T.; Morita, T. Tailor-Made Mannosylerythritol Lipids: Current State and Perspectives. *Appl Microbiol Biotechnol* **2018**, *102*, 6877–6884, doi:10.1007/s00253-018-9160-9.
168. Ceresa, C.; Hutton, S.; Lajarin-Cuesta, M.; Heaton, R.; Hargreaves, I.; Fracchia, L.; De Rienzo, M.A.D. Production of Mannosylerythritol Lipids (MELs) to Be Used as Antimicrobial Agents Against S. Aureus ATCC 6538. *Curr Microbiol* **2020**, *77*, 1373–1380, doi:10.1007/s00284-020-01927-2.
169. Niu, Y.; Wu, J.; Wang, W.; Chen, Q. Production and Characterization of a New Glycolipid, Mannosylerythritol Lipid, from Waste Cooking Oil Biotransformation by Pseudozyma Aphidis ZJUDM34. *Food Sci Nutr* **2019**, *7*, 937–948, doi:10.1002/fsn3.880.
170. Cui, Y.; Li, C.; Bao, M. Deep Eutectic Solvents (DESs) as Powerful and Recyclable Catalysts and Solvents for the Synthesis of 3,4-Dihydropyrimidin-2(1H)-Ones/Thiones. *Green Processing and Synthesis* **2019**, *8*, 568–576, doi:10.1515/gps-2019-0026.

171. Liang, X.; Fu, Y.; Chang, J. Effective Separation, Recovery and Recycling of Deep Eutectic Solvent after Biomass Fractionation with Membrane-Based Methodology. *Separation and Purification Technology* **2019**, *210*, 409–416, doi:10.1016/j.seppur.2018.08.021.
172. Jeong, K.M.; Lee, M.S.; Nam, M.W.; Zhao, J.; Jin, Y.; Lee, D.-K.; Kwon, S.W.; Jeong, J.H.; Lee, J. Tailoring and Recycling of Deep Eutectic Solvents as Sustainable and Efficient Extraction Media. *Journal of Chromatography A* **2015**, *1424*, 10–17, doi:10.1016/j.chroma.2015.10.083.
173. Fouladi, P.; Salamat, R.; A, A.; Aghdam Shahryar, H.; A, N. Effect of Choline Chloride Supplement on the Internal Organs and Carcass Weight of Broilers Chickens. *Journal of Animal and Veterinary Advances* **2008**, *7*.
174. Sclapari, T.; Bramati, V. New Uses of Choline Chloride in Agrochemical Formulations 2012.
175. Seyler, C.; Capello, C.; Hellweg, S.; Bruder, C.; Bayne, D.; Huwiler, A.; Hungerbühler, K. Waste-Solvent Management as an Element of Green Chemistry: A Comprehensive Study on the Swiss Chemical Industry. *Ind. Eng. Chem. Res.* **2006**, *45*, 7700–7709, doi:10.1021/ie060525l.
176. Abbott, B.J. Preparation of Pharmaceutical Compounds by Immobilized Enzymes and Cells. In *Advances in Applied Microbiology*; Perlman, D., Ed.; Academic Press, 1976; Vol. 20, pp. 203–257.
177. B. Ansorge-Schumacher, M.; Thum, O. Immobilised Lipases in the Cosmetics Industry. *Chemical Society Reviews* **2013**, *42*, 6475–6490, doi:10.1039/C3CS35484A.
178. Basso, A.; Serban, S. Industrial Applications of Immobilized Enzymes—A Review. *Molecular Catalysis* **2019**, *479*, 110607, doi:10.1016/j.mcat.2019.110607.
179. Hassan, M.E.; Yang, Q.; Xiao, Z.; Liu, L.; Wang, N.; Cui, X.; Yang, L. Impact of Immobilization Technology in Industrial and Pharmaceutical Applications. *3 Biotech* **2019**, *9*, doi:10.1007/s13205-019-1969-0.
180. Korupp, C.; Weberskirch, R.; Müller, J.J.; Liese, A.; Hilterhaus, L. Scaleup of Lipase-Catalyzed Polyester Synthesis. *Org. Process Res. Dev.* **2010**, *14*, 1118–1124, doi:10.1021/op1000868.
181. Wiemann, L.O.; Nieguth, R.; Eckstein, M.; Naumann, M.; Thum, O.; Ansorge-Schumacher, M.B. Composite Particles of Novozyme 435 and Silicone: Advancing Technical Applicability of Macroporous Enzyme Carriers. *ChemCatChem* **2009**, *1*, 455–462, doi:https://doi.org/10.1002/cctc.200900199.
182. Ansorge-Schumacher, M.B.; Nieguth, R.; Wiemann, L.O.; Weißhaupt, P.; Thum, O.; Eckstein, M. Belastbare Enzympräparate für die technische Biokatalyse. *Chemie Ingenieur Technik* **2010**, *82*, 35–42, doi:https://doi.org/10.1002/cite.200900105.
183. Keng, P.S.; Basri, M.; Ariff, A.B.; Abdul Rahman, M.B.; Abdul Rahman, R.N.Z.; Salleh, A.B. Scale-up Synthesis of Lipase-Catalyzed Palm Esters in Stirred-Tank Reactor. *Bioresource Technology* **2008**, *99*, 6097–6104, doi:10.1016/j.biortech.2007.12.049.
184. Paiva, A.; Matias, A.A.; Duarte, A.R.C. How Do We Drive Deep Eutectic Systems towards an Industrial Reality? *Current Opinion in Green and Sustainable Chemistry* **2018**, *11*, 81–85, doi:10.1016/j.cogsc.2018.05.010.
185. Pithani, S.; Karlsson, S.; Emtenäs, H.; Öberg, C.T. Using Spinchem Rotating Bed Reactor Technology for Immobilized Enzymatic Reactions: A Case Study. *Org. Process Res. Dev.* **2019**, *23*, 1926–1931, doi:10.1021/acs.oprd.9b00240.
186. Guajardo, N.; Schrebler, R.A.; Domínguez de María, P. From Batch to Fed-Batch and to Continuous Packed-Bed Reactors: Lipase-Catalyzed Esterifications in Low Viscous Deep-Eutectic-Solvents with Buffer as Cosolvent. *Bioresource Technology* **2019**, *273*, 320–325, doi:10.1016/j.biortech.2018.11.026.

187. Delavault, A.; Opochenska, O.; Laneque, L.; Soergel, H.; Muhle-Goll, C.; Ochsenreither, K.; Syldatk, C. Lipase-Catalyzed Production of Sorbitol Laurate in a "2-in-1" Deep Eutectic System: Factors Affecting the Synthesis and Scalability. *Molecules* **2021**, *26*, 2759, doi:10.3390/molecules26092759.
188. Francisco, M.; van den Bruinhorst, A.; Kroon, M.C. Low-Transition-Temperature Mixtures (LTTMs): A New Generation of Designer Solvents. *Angewandte Chemie International Edition* **2013**, *52*, 3074–3085, doi:10.1002/anie.201207548.
189. Kottaras, P.; Koulianos, M.; Makris, D. Low-Transition Temperature Mixtures (LTTMs) Made of Bioorganic Molecules: Enhanced Extraction of Antioxidant Phenolics from Industrial Cereal Solid Wastes. *Recycling* **2017**, *2*, 3, doi:10.3390/recycling2010003.
190. Rodríguez, N.R.; González, A.S.B.; Tijssen, P.M.A.; Kroon, M.C. Low Transition Temperature Mixtures (LTTMs) as Novel Entrainers in Extractive Distillation. *Fluid Phase Equilibria* **2015**, *385*, 72–78, doi:10.1016/j.fluid.2014.10.044.
191. Tang, B.; Row, K.H. Recent Developments in Deep Eutectic Solvents in Chemical Sciences. *Monatshefte für Chemie - Chemical Monthly* **2013**, *144*, 1427–1454, doi:10.1007/s00706-013-1050-3.
192. Priecel, P.; Lopez-Sanchez, J.A. Advantages and Limitations of Microwave Reactors: From Chemical Synthesis to the Catalytic Valorization of Biobased Chemicals. *ACS Sustainable Chem. Eng.* **2019**, *7*, 3–21, doi:10.1021/acssuschemeng.8b03286.
193. Khan, N.R.; Rathod, V.K. Microwave Assisted Enzymatic Synthesis of Speciality Esters: A Mini - Review. *Process Biochemistry* **2018**, *75*, 89–98, doi:10.1016/j.procbio.2018.08.019.
194. Yadav, G.D.; Lathi, P.S. Synergism between Microwave and Enzyme Catalysis in Intensification of Reactions and Selectivities: Transesterification of Methyl Acetoacetate with Alcohols. *Journal of Molecular Catalysis A: Chemical* **2004**, *223*, 51–56, doi:10.1016/j.molcata.2003.09.050.
195. Yadav, G.D.; Sajgure, A.D. Synergism of Microwave Irradiation and Enzyme Catalysis in Synthesis of Isoniazid. *Journal of Chemical Technology & Biotechnology* **2007**, *82*, 964–970, doi:https://doi.org/10.1002/jctb.1738.
196. Yadav, G.D.; Lathi, P.S. Synergism of Microwaves and Immobilized Enzyme Catalysis in Synthesis of Adipic Acid Esters in Nonaqueous Media. *Synthetic Communications* **2005**, *35*, 1699–1705, doi:10.1081/SCC-200061687.
197. Kuo, C.-H.; Chen, H.-H.; Chen, J.-H.; Liu, Y.-C.; Shieh, C.-J. High Yield of Wax Ester Synthesized from Cetyl Alcohol and Octanoic Acid by Lipozyme RMIM and Novozym 435. *International Journal of Molecular Sciences* **2012**, *13*, 11694–11704, doi:10.3390/ijms130911694.
198. Yadav, G.D.; Borkar, I.V. Kinetic and Mechanistic Investigation of Microwave-Assisted Lipase Catalyzed Synthesis of Citronellyl Acetate. *Ind. Eng. Chem. Res.* **2009**, *48*, 7915–7922, doi:10.1021/ie800591c.
199. Kondamudi, N.; McDougal, O.M. Microwave-Assisted Synthesis and Characterization of Stearic Acid Sucrose Ester: A Bio-Based Surfactant. *Journal of Surfactants and Detergents* **2019**, *22*, 721–729, doi:https://doi.org/10.1002/jsde.12280.
200. Leonelli, C.; Mason, T.J. Microwave and Ultrasonic Processing: Now a Realistic Option for Industry. *Chemical Engineering and Processing: Process Intensification* **2010**, *49*, 885–900, doi:10.1016/j.cep.2010.05.006.
201. Dange, P.N.; Rathod, V.K. Equilibrium and Thermodynamic Parameters for Heterogeneous Esterification of Butyric Acid with Methanol under Microwave Irradiation. *Resource-Efficient Technologies* **2017**, *3*, 64–70, doi:10.1016/j.reffit.2016.11.012.

202. Hoz, A. de la; Díaz-Ortiz, Á.; Moreno, A. Microwaves in Organic Synthesis. Thermal and Non-Thermal Microwave Effects. *Chem. Soc. Rev.* **2005**, *34*, 164–178, doi:10.1039/B411438H.
203. Yadav, G.D.; Thorat, P.A. Microwave Assisted Lipase Catalyzed Synthesis of Isoamyl Myristate in Solvent-Free System. *Journal of Molecular Catalysis B: Enzymatic* **2012**, *83*, 16–22, doi:10.1016/j.molcatb.2012.06.011.
204. Rathi, A.K.; Gawande, M.B.; Zboril, R.; Varma, R.S. Microwave-Assisted Synthesis – Catalytic Applications in Aqueous Media. *Coordination Chemistry Reviews* **2015**, *291*, 68–94, doi:10.1016/j.ccr.2015.01.011.
205. García, S.; Zhang, L.; Piburn, G.W.; Henkelman, G.; Humphrey, S.M. Microwave Synthesis of Classically Immiscible Rhodium–Silver and Rhodium–Gold Alloy Nanoparticles: Highly Active Hydrogenation Catalysts. *ACS Nano* **2014**, *8*, 11512–11521, doi:10.1021/nn504746u.
206. Flores, M.V.; Naraghi, K.; Engasser, J.-M.; Halling, P.J. Influence of Glucose Solubility and Dissolution Rate on the Kinetics of Lipase Catalyzed Synthesis of Glucose Laurate in 2-Methyl 2-Butanol. *Biotechnology and Bioengineering* **2002**, *78*, 815–821, doi:https://doi.org/10.1002/bit.10263.
207. Khaled, N.; Montet, D.; Pina, M.; Graille, J. Fructose Oleate Synthesis in a Fixed Catalyst Bed Reactor. *Biotechnology Letters* **1991**, *13*, 167–172, doi:10.1007/BF01025812.
208. Li, C.; Tan, T.; Zhang, H.; Feng, W. Analysis of the Conformational Stability and Activity of Candida Antarctica Lipase B in Organic Solvents: Insight from Molecular Dynamics and Quantum Mechanics/Simulations. *J Biol Chem* **2010**, *285*, 28434–28441, doi:10.1074/jbc.M110.136200.
209. Marathe, S.J.; Shah, N.N.; Singhal, R.S. Enzymatic Synthesis of Fatty Acid Esters of Trehalose: Process Optimization, Characterization of the Esters and Evaluation of Their Bioactivities. *Bioorganic Chemistry* **2020**, *94*, 103460, doi:10.1016/j.bioorg.2019.103460.
210. Espino, M.; de los Ángeles Fernández, M.; Gomez, F.J.V.; Silva, M.F. Natural Designer Solvents for Greening Analytical Chemistry. *TrAC Trends in Analytical Chemistry* **2016**, *76*, 126–136, doi:10.1016/j.trac.2015.11.006.
211. Gomez, F.J.V.; Espino, M.; de Los Angeles Fernandez, M.; Raba, J.; Silva, M.F. Enhanced Electrochemical Detection of Quercetin by Natural Deep Eutectic Solvents. *Anal Chim Acta* **2016**, *936*, 91–96, doi:10.1016/j.aca.2016.07.022.
212. Réjasse, B.; Lamare, S.; Legoy, M.-D.; Besson, T. Stability Improvement of Immobilized Candida Antarctica Lipase B in an Organic Medium under Microwave Radiation. *Organic & Biomolecular Chemistry* **2004**, *2*, 1086–1089, doi:10.1039/B401145G.
213. Réjasse, B.; Besson, T.; Legoy, M.-D.; Lamare, S. Influence of Microwave Radiation on Free Candida Antarctica Lipase B Activity and Stability. *Org. Biomol. Chem.* **2006**, *4*, 3703–3707, doi:10.1039/B610265D.
214. Martinez-Guerra, E.; Gude, V.G. Synergistic Effect of Simultaneous Microwave and Ultrasound Irradiations on Transesterification of Waste Vegetable Oil. *Fuel* **2014**, *137*, 100–108, doi:10.1016/j.fuel.2014.07.087.
215. Zhang, Y.; Xia, X.; Duan, M.; Han, Y.; Liu, J.; Luo, M.; Zhao, C.; Zu, Y.; Fu, Y. Green Deep Eutectic Solvent Assisted Enzymatic Preparation of Biodiesel from Yellow Horn Seed Oil with Microwave Irradiation. *Journal of Molecular Catalysis B: Enzymatic* **2016**, *123*, 35–40, doi:10.1016/j.molcatb.2015.10.013.
216. González-Rivera, J.; Husanu, E.; Mero, A.; Ferrari, C.; Duce, C.; Tinè, M.R.; D'Andrea, F.; Pomelli, C.S.; Guazzelli, L. Insights into Microwave Heating Response

and Thermal Decomposition Behavior of Deep Eutectic Solvents. *Journal of Molecular Liquids* **2020**, *300*, 112357, doi:10.1016/j.molliq.2019.112357.
217. Weaver, K.D.; Kim, H.J.; Sun, J.; MacFarlane, D.R.; Elliott, G.D. Cyto-Toxicity and Biocompatibility of a Family of Choline Phosphate Ionic Liquids Designed for Pharmaceutical Applications. *Green Chem.* **2010**, *12*, 507–513, doi:10.1039/B918726J.
218. Sontakke, J.B.; Yadav, G.D. Optimization and Kinetic Modeling of Lipase Catalyzed Enantioselective N-Acetylation of (±)-1-Phenylethylamine under Microwave Irradiation. *Journal of Chemical Technology & Biotechnology* **2011**, *86*, 739–748, doi:https://doi.org/10.1002/jctb.2582.
219. Loupy, A.; Perreux, L.; Liagre, M.; Burle, K.; Moneuse, M. Reactivity and selectivity under microwaves in organic chemistry. Relation with medium effects and reaction mechanisms. *Pure and Applied Chemistry* **2001**, *73*, 161–166, doi:10.1351/pac200173010161.
220. Naushad, M.; Alothman, Z.A.; Khan, A.B.; Ali, M. Effect of Ionic Liquid on Activity, Stability, and Structure of Enzymes: A Review. *Int J Biol Macromol* **2012**, *51*, 555–560, doi:10.1016/j.ijbiomac.2012.06.020.
221. Sanghamitra, N.J.M.; Ueno, T. Stability and Activity of Enzymes in Ionic Liquids. In *Green Solvents II: Properties and Applications of Ionic Liquids*; Mohammad, A., Inamuddin, Dr., Eds.; Springer Netherlands: Dordrecht, 2012; pp. 235–273 ISBN 978-94-007-2891-2.
222. Elgharbawy, A.A.; Hayyan, A.; Hayyan, M.; Rashid, S.N.; Nor, M.R.M.; Zulkifli, M.Y.; Alias, Y.; Mirghani, M.E.S. Shedding Light on Lipase Stability in Natural Deep Eutectic Solvents. *Chemical and Biochemical Engineering Quarterly* **2018**, *32*, 359–370, doi:10.15255/CABEQ.2018.1335.
223. Elversson, J.; Millqvist-Fureby, A. In Situ Coating--an Approach for Particle Modification and Encapsulation of Proteins during Spray-Drying. *Int J Pharm* **2006**, *323*, 52–63, doi:10.1016/j.ijpharm.2006.05.066.
224. Morschhäuser, R.; Krull, M.; Kayser, C.; Boberski, C.; Bierbaum, R.; Püschner, P.A.; Glasnov, T.N.; Kappe, C.O. Microwave-Assisted Continuous Flow Synthesis on Industrial Scale. *Green Processing and Synthesis* **2012**, *1*, 281–290, doi:10.1515/gps-2012-0032.
225. Pramod, K.; Kotta, S.; Jijith, U.S.; Aravind, A.; Abu Tahir, M.; Manju, C.S.; Gangadharappa, H.V. Surfactant-Based Prophylaxis and Therapy against COVID-19: A Possibility. *Medical Hypotheses* **2020**, *143*, 110081, doi:10.1016/j.mehy.2020.110081.
226. Gallou, F.; A. Isley, N.; Ganic, A.; Onken, U.; Parmentier, M. Surfactant Technology Applied toward an Active Pharmaceutical Ingredient: More than a Simple Green Chemistry Advance. *Green Chemistry* **2016**, *18*, 14–19, doi:10.1039/C5GC02371H.
227. Baccile, N.; Nassif, N.; Malfatti, L.; Bogaert, I.N.A.V.; Soetaert, W.; Pehau-Arnaudet, G.; Babonneau, F. Sophorolipids: A Yeast-Derived Glycolipid as Greener Structure Directing Agents for Self-Assembled Nanomaterials. *Green Chemistry* **2010**, *12*, 1564–1567, doi:10.1039/C0GC00163E.
228. Hirata, Y.; Ryu, M.; Oda, Y.; Igarashi, K.; Nagatsuka, A.; Furuta, T.; Sugiura, M. Novel Characteristics of Sophorolipids, Yeast Glycolipid Biosurfactants, as Biodegradable Low-Foaming Surfactants. *Journal of Bioscience and Bioengineering* **2009**, *108*, 142–146, doi:10.1016/j.jbiosc.2009.03.012.
229. Davies, E.; Deutz, P.; Zein, S.H. Single-Step Extraction-Esterification Process to Produce Biodiesel from Palm Oil Mill Effluent (POME) Using Microwave Heating: A Circular Economy Approach to Making Use of a Difficult Waste Product. *Biomass Conversion and Biorefinery* **2020**, doi:10.1007/s13399-020-00856-1.

230. Abbott, A.P.; Boothby, D.; Capper, G.; Davies, D.L.; Rasheed, R.K. Deep Eutectic Solvents Formed between Choline Chloride and Carboxylic Acids: Versatile Alternatives to Ionic Liquids. *Journal of the American Chemical Society* **2004**, *126*, 9142–9147, doi:10.1021/ja048266j.
231. Gorte, O.; Hollenbach, R.; Papachristou, I.; Steinweg, C.; Silve, A.; Frey, W.; Syldatk, C.; Ochsenreither, K. Evaluation of Downstream Processing, Extraction, and Quantification Strategies for Single Cell Oil Produced by the Oleaginous Yeasts Saitozyma Podzolica DSM 27192 and Apiotrichum Porosum DSM 27194. *Front. Bioeng. Biotechnol.* **2020**, *8*, 355, doi:10.3389/fbioe.2020.00355.
232. Ochsenreither, K.; Glück, C.; Stressler, T.; Fischer, L.; Syldatk, C. Production Strategies and Applications of Microbial Single Cell Oils. *Front. Microbiol.* **2016**, *7*, doi:10.3389/fmicb.2016.01539.
233. Folch, J.; Lees, M.; Sloane Stanley, G.H. A Simple Method for the Isolation and Purification of Total Lipides from Animal Tissues. *J Biol Chem* **1957**, *226*, 497–509.
234. Liauw, M.Y.; Natan, F.A.; Widiyanti, P.; Ikasari, D.; Indraswati, N.; Soetaredjo, F.E. *EXTRACTION OF NEEM OIL (Azadirachta Indica A. Juss) USING N-HEXANE AND ETHANOL: STUDIES OF OIL QUALITY, KINETIC AND THERMODYNAMIC*;
235. Schulze, I.; Hansen, S.; Großhans, S.; Rudszuck, T.; Ochsenreither, K.; Syldatk, C.; Neumann, A. Characterization of Newly Isolated Oleaginous Yeasts - Cryptococcus Podzolicus, Trichosporon Porosum and Pichia Segobiensis. *AMB Expr* **2014**, *4*, 24, doi:10.1186/s13568-014-0024-0.
236. Aliyu, H.; Gorte, O.; Neumann, A.; Ochsenreither, K. Draft Genome Sequence of the Oleaginous Yeast Saitozyma Podzolica (Syn. Cryptococcus Podzolicus) DSM 27192. *Microbiol Resour Announc* **2019**, *8*, doi:10.1128/MRA.01676-18.
237. Dallinger, D.; Kappe, C.O. Microwave-Assisted Synthesis in Water as Solvent. *Chem. Rev.* **2007**, *107*, 2563–2591, doi:10.1021/cr0509410.
238. Microwave Theory. In *Microwaves in Organic and Medicinal Chemistry*; John Wiley & Sons, Ltd, 2012; pp. 9–39 ISBN 978-3-527-64782-8.
239. LidstroÈm, P.; Tierney, J.; Wathey, B.; Westman, J. Microwave Assisted Organic SynthesisÐa Review. **2001**, 59.
240. Nie, J.; Yu, G.; Song, Z.; Wang, X.; Li, Z.; She, Y.; Lee, M. Microwave-Assisted Deep Eutectic Solvent Extraction Coupled with Headspace Solid-Phase Microextraction Followed by GC-MS for the Analysis of Volatile Compounds from Tobacco. *Anal. Methods* **2017**, *9*, 856–863, doi:10.1039/C6AY03076A.
241. Herrero, M.A.; Kremsner, J.M.; Kappe, C.O. Nonthermal Microwave Effects Revisited: On the Importance of Internal Temperature Monitoring and Agitation in Microwave Chemistry. *J. Org. Chem.* **2008**, *73*, 36–47, doi:10.1021/jo7022697.
242. Tasei, Y.; Mijiddorj, B.; Fujito, T.; Kawamura, I.; Ueda, K.; Naito, A. Thermal and Nonthermal Microwave Effects of Ethanol and Hexane-Mixed Solution as Revealed by In Situ Microwave Irradiation Nuclear Magnetic Resonance Spectroscopy and Molecular Dynamics Simulation. *J. Phys. Chem. B* **2020**, *124*, 9615–9624, doi:10.1021/acs.jpcb.0c06383.
243. Kubo, M.T.; Siguemoto, É.S.; Funcia, E.S.; Augusto, P.E.; Curet, S.; Boillereaux, L.; Sastry, S.K.; Gut, J.A. Non-Thermal Effects of Microwave and Ohmic Processing on Microbial and Enzyme Inactivation: A Critical Review. *Current Opinion in Food Science* **2020**, *35*, 36–48, doi:10.1016/j.cofs.2020.01.004.
244. Coultate, T.P. Polysaccharides. In *Food*; 2001; pp. 41–72.
245. Jiang, B.; Liu, Y.; Bhandari, B.; Zhou, W. Impact of Caramelization on the Glass Transition Temperature of Several Caramelized Sugars. Part I: Chemical Analyses. *J Agric Food Chem* **2008**, *56*, 5138–5147, doi:10.1021/jf703791e.

246. Aroso, I.M.; Paiva, A.; Reis, R.L.; Duarte, A.R.C. Natural Deep Eutectic Solvents from Choline Chloride and Betaine – Physicochemical Properties. *Journal of Molecular Liquids* **2017**, *241*, 654–661, doi:10.1016/j.molliq.2017.06.051.
247. Xu, M.; Ran, L.; Chen, N.; Fan, X.; Ren, D.; Yi, L. Polarity-Dependent Extraction of Flavonoids from Citrus Peel Waste Using a Tailor-Made Deep Eutectic Solvent. *Food Chem* **2019**, *297*, 124970, doi:10.1016/j.foodchem.2019.124970.
248. Sathish, A.; Smith, B.R.; Sims, R.C. Effect of Moisture on in Situ Transesterification of Microalgae for Biodiesel Production. *Journal of Chemical Technology & Biotechnology* **2014**, *89*, 137–142, doi:https://doi.org/10.1002/jctb.4125.
249. Yousuf, A.; Khan, M.R.; Islam, M.A.; Wahid, Z.A.; Pirozzi, D. Technical Difficulties and Solutions of Direct Transesterification Process of Microbial Oil for Biodiesel Synthesis. *Biotechnology Letters* **2016**, doi:10.1007/s10529-016-2217-x.
250. Jin, G.; Yang, F.; Hu, C.; Shen, H.; Zhao, Z.K. Enzyme-Assisted Extraction of Lipids Directly from the Culture of the Oleaginous Yeast Rhodosporidium Toruloides. *Bioresour Technol* **2012**, *111*, 378–382, doi:10.1016/j.biortech.2012.01.152.
251. Klei, I.V.D.; Veenhuis, M.; Brul, S.; Klis, F.M.; Groot, P.W.J.D.; Müller, W.H.; Driel, K.G.A. van; Boekhout, T. Cytology, Cell Walls and Septa: A Summary of Yeast Cell Biology from a Phylogenetic Perspective. *The Yeasts* **2011**, 111–128, doi:10.1016/B978-0-444-52149-1.00008-2.
252. Khoomrung, S.; Chumnanpuen, P.; Jansa-Ard, S.; Ståhlman, M.; Nookaew, I.; Borén, J.; Nielsen, J. Rapid Quantification of Yeast Lipid Using Microwave-Assisted Total Lipid Extraction and HPLC-CAD. *Anal Chem* **2013**, *85*, 4912–4919, doi:10.1021/ac3032405.
253. Lee, J.-Y.; Yoo, C.; Jun, S.-Y.; Ahn, C.-Y.; Oh, H.-M. Comparison of Several Methods for Effective Lipid Extraction from Microalgae. *Bioresource Technology* **2010**, *101*, S75–S77, doi:10.1016/j.biortech.2009.03.058.
254. Tommasi, E.; Cravotto, G.; Galletti, P.; Grillo, G.; Mazzotti, M.; Sacchetti, G.; Samorì, C.; Tabasso, S.; Tacchini, M.; Tagliavini, E. Enhanced and Selective Lipid Extraction from the Microalga P. Tricornutum by Dimethyl Carbonate and Supercritical CO2 Using Deep Eutectic Solvents and Microwaves as Pretreatment. *ACS Sustainable Chem. Eng.* **2017**, *5*, 8316–8322, doi:10.1021/acssuschemeng.7b02074.
255. Martins, A.B.; Graebin, N.G.; Lorenzoni, A.S.G.; Fernandez-Lafuente, R.; Ayub, M.A.Z.; Rodrigues, R.C. Rapid and High Yields of Synthesis of Butyl Acetate Catalyzed by Novozym 435: Reaction Optimization by Response Surface Methodology. *Process Biochemistry* **2011**, *46*, 2311–2316, doi:10.1016/j.procbio.2011.09.011.
256. Dai, Y.; van Spronsen, J.; Witkamp, G.-J.; Verpoorte, R.; Choi, Y.H. Natural Deep Eutectic Solvents as New Potential Media for Green Technology. *Analytica Chimica Acta* **2013**, *766*, 61–68, doi:10.1016/j.aca.2012.12.019.
257. Lieu, T.; Yusup, S.; Moniruzzaman, M. Kinetic Study on Microwave-Assisted Esterification of Free Fatty Acids Derived from Ceiba Pentandra Seed Oil. *Bioresource Technology* **2016**, *211*, 248–256, doi:10.1016/j.biortech.2016.03.105.
258. Reyes, H.R.; Hill, C.G. Kinetic Modeling of Interesterification Reactions Catalyzed by Immobilized Lipase. *Biotechnology and Bioengineering* **1994**, *43*, 171–182, doi:https://doi.org/10.1002/bit.260430211.
259. Keramat, M.; Golmakani, M.-T.; Durand, E.; Villeneuve, P.; Hosseini, S.M.H. Auto-Catalytic Production of Eugenyl Acetate and Eugenyl Butyrate Using Microwave Radiation: A Kinetic and Mechanism-Related Approach. *Journal of Chemical Technology & Biotechnology n/a*, doi:https://doi.org/10.1002/jctb.6583.

260. Kim, Y.-H.; Choi, Y.-K.; Park, J.; Lee, S.; Yang, Y.-H.; Kim, H.J.; Park, T.-J.; Hwan Kim, Y.; Lee, S.H. Ionic Liquid-Mediated Extraction of Lipids from Algal Biomass. *Bioresource Technology* **2012**, *109*, 312–315, doi:10.1016/j.biortech.2011.04.064.
261. Ganske, F.; Bornscheuer, U.T. Lipase-Catalyzed Glucose Fatty Acid Ester Synthesis in Ionic Liquids. *Org. Lett.* **2005**, *7*, 3097–3098, doi:10.1021/ol0511169.
262. Radošević, K.; Cvjetko Bubalo, M.; Gaurina Srček, V.; Grgas, D.; Landeka Dragičević, T.; Radojčić Redovniković, I. Evaluation of Toxicity and Biodegradability of Choline Chloride Based Deep Eutectic Solvents. *Ecotoxicology and Environmental Safety* **2015**, *112*, 46–53, doi:10.1016/j.ecoenv.2014.09.034.
263. Durand, E.; Lecomte, J.; Baréa, B.; Piombo, G.; Dubreucq, E.; Villeneuve, P. Evaluation of Deep Eutectic Solvents as New Media for Candida Antarctica B Lipase Catalyzed Reactions. *Process Biochemistry* **2012**, *47*, 2081–2089, doi:10.1016/j.procbio.2012.07.027.
264. Bermúdez, J.M.; Beneroso, D.; Rey-Raap, N.; Arenillas, A.; Menéndez, J.A. Energy Consumption Estimation in the Scaling-up of Microwave Heating Processes. *Chemical Engineering and Processing: Process Intensification* **2015**, *95*, 1–8, doi:10.1016/j.cep.2015.05.001.
265. Pöhnlein, M.; Hausmann, R.; Lang, S.; Syldatk, C. Enzymatic Synthesis and Modification of Surface-Active Glycolipids: Enz Syn and Mod of Glycolipids. *European Journal of Lipid Science and Technology* **2015**, *117*, 145–155, doi:10.1002/ejlt.201400418.
266. Ewald, N.; Vidakovic, A.; Langeland, M.; Kiessling, A.; Sampels, S.; Lalander, C. Fatty Acid Composition of Black Soldier Fly Larvae (Hermetia Illucens) – Possibilities and Limitations for Modification through Diet. *Waste Management* **2020**, *102*, 40–47, doi:10.1016/j.wasman.2019.10.014.
267. Hoc, B.; Genva, M.; Fauconnier, M.-L.; Lognay, G.; Francis, F.; Caparros Megido, R. About Lipid Metabolism in Hermetia Illucens (L. 1758): On the Origin of Fatty Acids in Prepupae. *Scientific Reports* **2020**, *10*, 11916, doi:10.1038/s41598-020-68784-8.
268. Szűts, A.; Szabó-Révész, P. Sucrose Esters as Natural Surfactants in Drug Delivery Systems--a Mini-Review. *Int J Pharm* **2012**, *433*, 1–9, doi:10.1016/j.ijpharm.2012.04.076.
269. Karlson, P.; Doenecke, D.; Koolman, J.; Fuchs, G.; Gerok, W. *Karlsons Biochemie und Pathobiochemie*; 15., vollst. überarb. u. neugestalt. Edition.; Thieme: Stuttgart, 2005; ISBN 978-3-13-357815-8.
270. Arcens, D.; Grau, E.; Grelier, S.; Cramail, H.; Peruch, F. Impact of Fatty Acid Structure on CALB-Catalyzed Esterification of Glucose. *Eur. J. Lipid Sci. Technol.* **2020**, *122*, 1900294, doi:10.1002/ejlt.201900294.
271. Jull, A.B.; Cullum, N.; Dumville, J.C.; Westby, M.J.; Deshpande, S.; Walker, N. Honey as a Topical Treatment for Wounds. *Cochrane Database of Systematic Reviews* **2015**, doi:10.1002/14651858.CD005083.pub4.
272. Cooper, R.A.; Molan, P.C.; Harding, K.G. Antibacterial Activity of Honey against Strains of Staphylococcus Aureus from Infected Wounds. *Journal of the royal society of medicine* **1999**, *92*, 283–285.
273. Weston, R.J.; Mitchell, K.R.; Allen, K.L. Antibacterial Phenolic Components of New Zealand Manuka Honey. *Food chemistry* **1999**, *64*, 295–301.
274. Simon, A.; Traynor, K.; Santos, K.; Blaser, G.; Bode, U.; Molan, P. Medical Honey for Wound Care—Still the 'Latest Resort'? *Evid Based Complement Alternat Med* **2009**, *6*, 165–173, doi:10.1093/ecam/nem175.
275. Mavric, E.; Wittmann, S.; Barth, G.; Henle, T. Identification and Quantification of Methylglyoxal as the Dominant Antibacterial Constituent of Manuka (Leptospermum

Scoparium) Honeys from New Zealand. *Mol Nutr Food Res* **2008**, *52*, 483–489, doi:10.1002/mnfr.200700282.
276. Degen, J.; Hellwig, M.; Henle, T. 1,2-Dicarbonyl Compounds in Commonly Consumed Foods. *Journal of Agricultural and Food Chemistry* **2012**, *60*, 7071–7079, doi:10.1021/jf301306g.
277. Arena, E.; Ballistreri, G.; Tomaselli, F.; Fallico, B. Survey of 1,2-Dicarbonyl Compounds in Commercial Honey of Different Floral Origin. *Journal of Food Science* **2011**, *76*, C1203–C1210, doi:10.1111/j.1750-3841.2011.02352.x.
278. Kwakman, P.H.S.; Boer, L.; Ruyter-Spira, C.P.; Creemers-Molenaar, T.; Helsper, J.P.F.G.; Vandenbroucke-Grauls, C.M.J.E.; Zaat, S.A.J.; Velde, A.A. Medical-Grade Honey Enriched with Antimicrobial Peptides Has Enhanced Activity against Antibiotic-Resistant Pathogens. *European Journal of Clinical Microbiology & Infectious Diseases* **2011**, *30*, 251–257, doi:10.1007/s10096-010-1077-x.
279. Siebenhaller, S.; Gentes, J.; Infantes, A.; Muhle-Goll, C.; Kirschhöfer, F.; Brenner-Weiß, G.; Ochsenreither, K.; Syldatk, C. Lipase-Catalyzed Synthesis of Sugar Esters in Honey and Agave Syrup. *Front Chem* **2018**, *6*, doi:10.3389/fchem.2018.00024.
280. Bornscheuer, U.; Herar, A.; Kreye, L.; Wendel, V.; Capewell, A.; Meyer, H.H.; Scheper, T.; Kolisis, F.N. Factors Affecting the Lipase Catalyzed Transesterification Reactions of 3-Hydroxy Esters in Organic Solvents. *Tetrahedron: Asymmetry* **1993**, *4*, 1007–1016, doi:10.1016/S0957-4166(00)80145-7.
281. Lee, K.P.; Kim, H.K. Antibacterial Effect of Fructose Laurate Synthesized by Candida Antarctica B Lipase-Mediated Transesterification. *Journal of Microbiology and Biotechnology* **2016**, *26*, 1579–1585, doi:10.4014/jmb.1601.01045.
282. Mandal, M.D.; Mandal, S. Honey: Its Medicinal Property and Antibacterial Activity. *Asian Pac J Trop Biomed* **2011**, *1*, 154–160, doi:10.1016/S2221-1691(11)60016-6.
283. Molan, P.C.; Rhodes, T. Honey: A Biologic Wound Dressing. **2015**.
284. Ventola, C.L. The Antibiotic Resistance Crisis. *P T* **2015**, *40*, 277–283.
285. Wright, G. Antibiotics: An Irresistible Newcomer. *Nature* **2015**, *517*, 442–444, doi:10.1038/nature14193.
286. Zoheir, A. *Stress Response and Adaptive Evolution of Bacteria in Microfluidic Systems*; Karlsruhe, 2020; p. 140;.
287. Landini, P.; Egli, T.; Wolf, J.; Lacour, S. SigmaS, a Major Player in the Response to Environmental Stresses in Escherichia Coli: Role, Regulation and Mechanisms of Promoter Recognition. *Environmental Microbiology Reports* **2014**, *6*, 1–13, doi:10.1111/1758-2229.12112.
288. Arsène, F.; Tomoyasu, T.; Bukau, B. The Heat Shock Response of Escherichia Coli. *International Journal of Food Microbiology* **2000**, *55*, 3–9, doi:10.1016/S0168-1605(00)00206-3.
289. Simmons, L.A.; Foti, J.J.; Cohen, S.E.; Walker, G.C. The SOS Regulatory Network. *EcoSal Plus* **2008**, *3*, doi:10.1128/ecosalplus.5.4.3.
290. Power, D.A.; Zimbro, M.J. *Difco & BBL Manual: Manual of Microbiological Culture Media*; Difco Laboratories, Division of Becton Dickinson and Co.: Sparks, MD, 2003; ISBN 978-0-9727207-0-0.
291. Patton, T.; Barrett, J.; Brennan, J.; Moran, N. Use of a Spectrophotometric Bioassay for Determination of Microbial Sensitivity to Manuka Honey. *Journal of Microbiological Methods* **2006**, *64*, 84–95, doi:10.1016/j.mimet.2005.04.007.
292. Russell, K.M.; Molan, P.C.; Wilkins, A.L.; Holland, P.T. Identification of Some Antibacterial Constituents of New Zealand Manuka Honey. *J. Agric. Food Chem.* **1990**, *38*, 10–13, doi:10.1021/jf00091a002.

293. Allen, K.L.; Molan, P.C.; Reid, G.M. A Survey of the Antibacterial Activity of Some New Zealand Honeys. *Journal of Pharmacy and Pharmacology* **1991**, *43*, 817–822, doi:10.1111/j.2042-7158.1991.tb03186.x.
294. Molan, P. Why Honey Is Effective as a Medicine. *Bee World* **2001**, *82*, 22–40, doi:10.1080/0005772X.2001.11099498.
295. Alvarez-Suarez, J.; Gasparrini, M.; Forbes-Hernández, T.; Mazzoni, L.; Giampieri, F. The Composition and Biological Activity of Honey: A Focus on Manuka Honey. *Foods* **2014**, *3*, 420–432, doi:10.3390/foods3030420.
296. Molan, P.C. The Antibacterial Activity of Honey. *Bee World* **1992**, *73*, 5–28, doi:10.1080/0005772X.1992.11099109.
297. Siebenhaller, S.; Grüninger, J.; Syldatk, C. Enzymatic Synthesis of Glycolipid Surfactants. In *Lipid Modification by Enzymes and Engineered Microbes*; Elsevier, 2018; pp. 293–313 ISBN 978-0-12-813167-1.
298. Adams, C.J.; Manley-Harris, M.; Molan, P.C. The Origin of Methylglyoxal in New Zealand Manuka (Leptospermum Scoparium) Honey. *Carbohydrate Research* **2009**, *344*, 1050–1053, doi:10.1016/j.carres.2009.03.020.
299. Grainger, M.N.C.; Manley-Harris, M.; Lane, J.R.; Field, R.J. Kinetics of Conversion of Dihydroxyacetone to Methylglyoxal in New Zealand Mānuka Honey: Part I – Honey Systems. *Food Chemistry* **2016**, *202*, 484–491, doi:10.1016/j.foodchem.2016.02.029.
300. Willix, D.J.; Molan, P.C.; Harfoot, C.G. A Comparison of the Sensitivity of Wound-Infecting Species of Bacteria to the Antibacterial Activity of Manuka Honey and Other Honey. *Journal of Applied Bacteriology* **1992**, *73*, 388–394, doi:10.1111/j.1365-2672.1992.tb04993.x.
301. Shaburova, O.V.; Krylov, S.V.; Veiko, V.P.; Pleteneva, E.A.; Burkal'tseva, M.V.; Miroshnikov, K.A.; Cornelissen, A.; Lavigne, R.; Sykilinda, N.N.; Kadykov, V.A.; et al. Search for Destruction Factors of Bacterial Biofilms: Comparison of Phage Properties in a Group of Pseudomonas Putida Bacteriophages and Specificity of Their Halo-Formation Products. *Russ J Genet* **2009**, *45*, 161–170, doi:10.1134/S1022795409020057.
302. Wagh, A.; Shen, S.; Shen, F.A.; Miller, C.D.; Walsh, M.K. Effect of Lactose Monolaurate on Pathogenic and Nonpathogenic Bacteria. *Appl. Environ. Microbiol.* **2012**, *78*, 3465–3468, doi:10.1128/AEM.07701-11.
303. Kulakovskaya, T.V.; Shashkov, A.S.; Kulakovskaya, E.V.; Golubev, W.I. Characterization of an Antifungal Glycolipid Secreted by the Yeast Sympodiomycopsis Paphiopedili. *FEMS Yeast Res* **2004**, *5*, 247–252, doi:10.1016/j.femsyr.2004.07.008.
304. Besada-Lombana, P.B.; Fernandez-Moya, R.; Fenster, J.; Da Silva, N.A. Engineering Saccharomyces Cerevisiae Fatty Acid Composition for Increased Tolerance to Octanoic Acid. *Biotechnology and Bioengineering* **2017**, *114*, 1531–1538, doi:10.1002/bit.26288.
305. Roelants, S.L.K.W.; Saerens, K.M.J.; Derycke, T.; Li, B.; Lin, Y.-C.; Peer, Y.V. de; Maeseneire, S.L.D.; Bogaert, I.N.A.V.; Soetaert, W. Candida Bombicola as a Platform Organism for the Production of Tailor-Made Biomolecules. *Biotechnology and Bioengineering* **2013**, *110*, 2494–2503, doi:10.1002/bit.24895.
306. Vakhlu, J. Yeast Lipases: Enzyme Purification, Biochemical Properties and Gene Cloning. *Electronic Journal of Biotechnology* **2006**, *9*, 0–0.
307. Manolakaki, D.; Velmahos, G.; Kourkoumpetis, T.; Chang, Y.; Alam, H.B.; Moya, M.M.D.; Mylonakis, E. Candida Infection and Colonization among Trauma Patients. *Virulence* **2010**, *1*, 367–375, doi:10.4161/viru.1.5.12796.
308. Turner, S.A.; Butler, G. The Candida Pathogenic Species Complex. *Cold Spring Harb Perspect Med* **2014**, *4*, a019778, doi:10.1101/cshperspect.a019778.

309. Donlan, R.M. Biofilm Formation: A Clinically Relevant Microbiological Process. *Clin Infect Dis* **2001**, *33*, 1387–1392, doi:10.1086/322972.
310. Ferrer, M.; Soliveri, J.; Plou, F.J.; López-Cortés, N.; Reyes-Duarte, D.; Christensen, M.; Copa-Patiño, J.L.; Ballesteros, A. Synthesis of Sugar Esters in Solvent Mixtures by Lipases from Thermomyces Lanuginosus and Candida Antarctica B, and Their Antimicrobial Properties. *Enzyme and Microbial Technology* **2005**, *36*, 391–398, doi:10.1016/j.enzmictec.2004.02.009.
311. Dusane, D.H.; Rajput, J.K.; Kumar, A.R.; Nancharaiah, Y.V.; Venugopalan, V.P.; Zinjarde, S.S. Disruption of Fungal and Bacterial Biofilms by Lauroyl Glucose. *Letters in Applied Microbiology* **2008**, *47*, 374–379, doi:10.1111/j.1472-765X.2008.02440.x.
312. Hollenbach, R.; Völp, A.R.; Höfert, L.; Rudat, J.; Ochsenreither, K.; Willenbacher, N.; Syldatk, C. Interfacial and Foaming Properties of Tailor-Made Glycolipids—Influence of the Hydrophilic Head Group and Functional Groups in the Hydrophobic Tail. *Molecules* **2020**, *25*, 3797, doi:10.3390/molecules25173797.
313. Wise, R.; Hart, T.; Cars, O.; Streulens, M.; Helmuth, R.; Huovinen, P.; Sprenger, M. Antimicrobial Resistance. *BMJ* **1998**, *317*, 609–610, doi:10.1136/bmj.317.7159.609.
314. Lambert, P.A. Bacterial Resistance to Antibiotics: Modified Target Sites. *Advanced Drug Delivery Reviews* **2005**, *57*, 1471–1485, doi:10.1016/j.addr.2005.04.003.
315. Tenover, F.C. Mechanisms of Antimicrobial Resistance in Bacteria. *The American Journal of Medicine* **2006**, *119*, S3–S10, doi:10.1016/j.amjmed.2006.03.011.
316. Aroso, I.M.; Silva, J.C.; Mano, F.; Ferreira, A.S.D.; Dionísio, M.; Sá-Nogueira, I.; Barreiros, S.; Reis, R.L.; Paiva, A.; Duarte, A.R.C. Dissolution Enhancement of Active Pharmaceutical Ingredients by Therapeutic Deep Eutectic Systems. *European Journal of Pharmaceutics and Biopharmaceutics* **2016**, *98*, 57–66, doi:10.1016/j.ejpb.2015.11.002.
317. Radošević, K.; Ćurko, N.; Gaurina Srček, V.; Cvjetko Bubalo, M.; Tomašević, M.; Kovačević Ganić, K.; Radojčić Redovniković, I. Natural Deep Eutectic Solvents as Beneficial Extractants for Enhancement of Plant Extracts Bioactivity. *LWT* **2016**, *73*, 45–51, doi:10.1016/j.lwt.2016.05.037.
318. Chen, J.; Wang, Q.; Liu, M.; Zhang, L. The Effect of Deep Eutectic Solvent on the Pharmacokinetics of Salvianolic Acid B in Rats and Its Acute Toxicity Test. *Journal of Chromatography B* **2017**, *1063*, 60–66, doi:10.1016/j.jchromb.2017.08.016.
319. Wikene, K.O.; Rukke, H.V.; Bruzell, E.; Tønnesen, H.H. Investigation of the Antimicrobial Effect of Natural Deep Eutectic Solvents (NADES) as Solvents in Antimicrobial Photodynamic Therapy. *Journal of Photochemistry and Photobiology B: Biology* **2017**, *171*, 27–33, doi:10.1016/j.jphotobiol.2017.04.030.

List of Figures

List of Tables

List of Abbreviations

AcDES	Acidified deep eutectic solvent
APG(s)	Alkylpolyglucoside(s)
C.	*Candida*
C10	Decanoic acid or ester
C12	Lauric acid or ester
C14	Myristic acid or ester
C16	Palmitic acid or ester
C8	Octanoic acid or ester
CDW	Cell dry weight
ChCl	Choline chloride
CMC	Critical micelle concentration
COSY	Correlated spectroscopy
D	Dimension
DES(s)	Deep eutectic solvent(s)
DNA	DeoxyriboNucleic Acid
DSM	Deutsche Sammlung von Mikroorganismen und Zellkulturen
DSP	Downstream processing
DT	Direct transesterification
E.	*Escherichia*
ELSD	Evaporative light scattering detector
EMK	Ethylmethylketone
EtAc	Ethyl acetate
FAME(s)	Fatty acid methyl ester(s)
FE	Folch extraction
GDH	Glycolipid decanoate honey

GLH	Glycolipid laurate honey
GMH	Glycolipid myristate honey
GOH	Glycolipid octanoate honey
GPH	Glycolipid palmitate honey
Gram–	Gram negative
Gram+	Gram positive
HBA	Hydrogen bond acceptor
HBD	Hydrogen bond donor
HCl	Hydrochloric acid
HMBC	Heteronuclear Multiple Bond Correlation
HPLC	High performance liquid chromatography
HSQC	Heteronuclear Single Quantum Coherence
iCalB	Immobilized lipase B from *Candida Antarctica*
IL(s)	Ionic liquid(s)
LB+kann	Luria-Bertani + kanamycin
Log P	Logarithm of the partition coefficient
MEL(s)	Mannosylerythritol lipid(s)
MGO	Methylglyoxal
MH	Manuka honey
MHWE	Manuka honey without enzyme
MRSA	Methicillin resistant staphylococcus aureus
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
OD_{600}	Optical density at 600 nm
OFaaT	One factor at a time
P.	*Pseudomonas*
Rf	Retention factor

RGB-S	Red green blue-strain
S.	*Saitozyma*
SE	Sugar esters
SL	Sorbitol laurate
Std	Standard
STR	Stirred tank reactor
TLC	Thin layer chromatography
TOCSY	Total correlation spectroscopy
VL	Vinyl laurate

Appendix

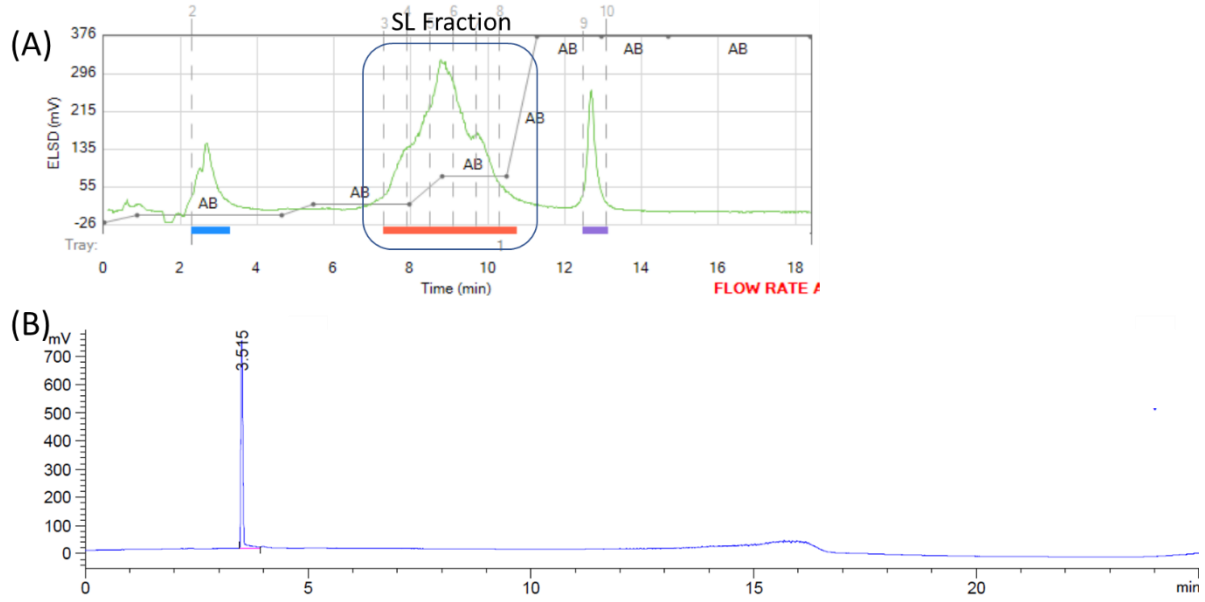


Figure A1. (A) Chromatogram of the flash-chromatography purification of sorbitol-6-O-laurate. (B) Chromatogram for the purity assessment of the resulting sorbitol-6-O-laurate standard.

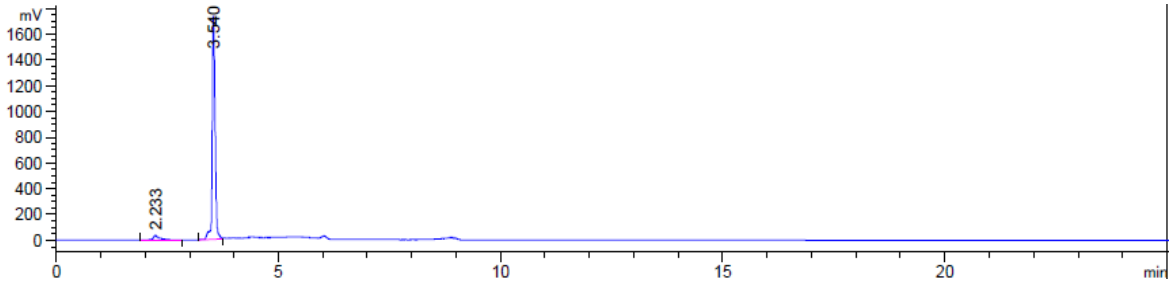


Figure A2. Chromatogram for the purity assessment of the resulting sorbitol-6-O-laurate from the STR production using liquid-liquid extraction for DSP.

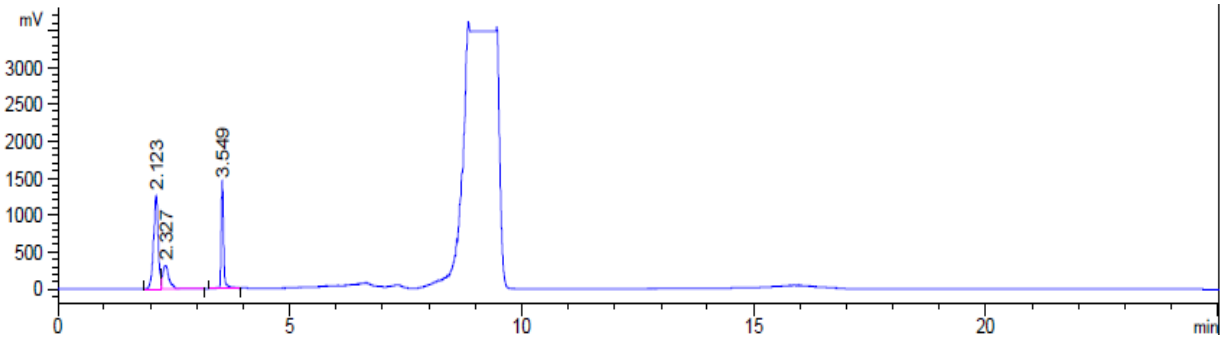


Figure A3. Chromatogram of HPLC-ELSD separation of extracts from glycolipid synthesis. At 2.1 min elutes the sorbitol, at 3.6min the sorbitol-6-O-laurate and around 9.5 min the lauric acid (not integrated).

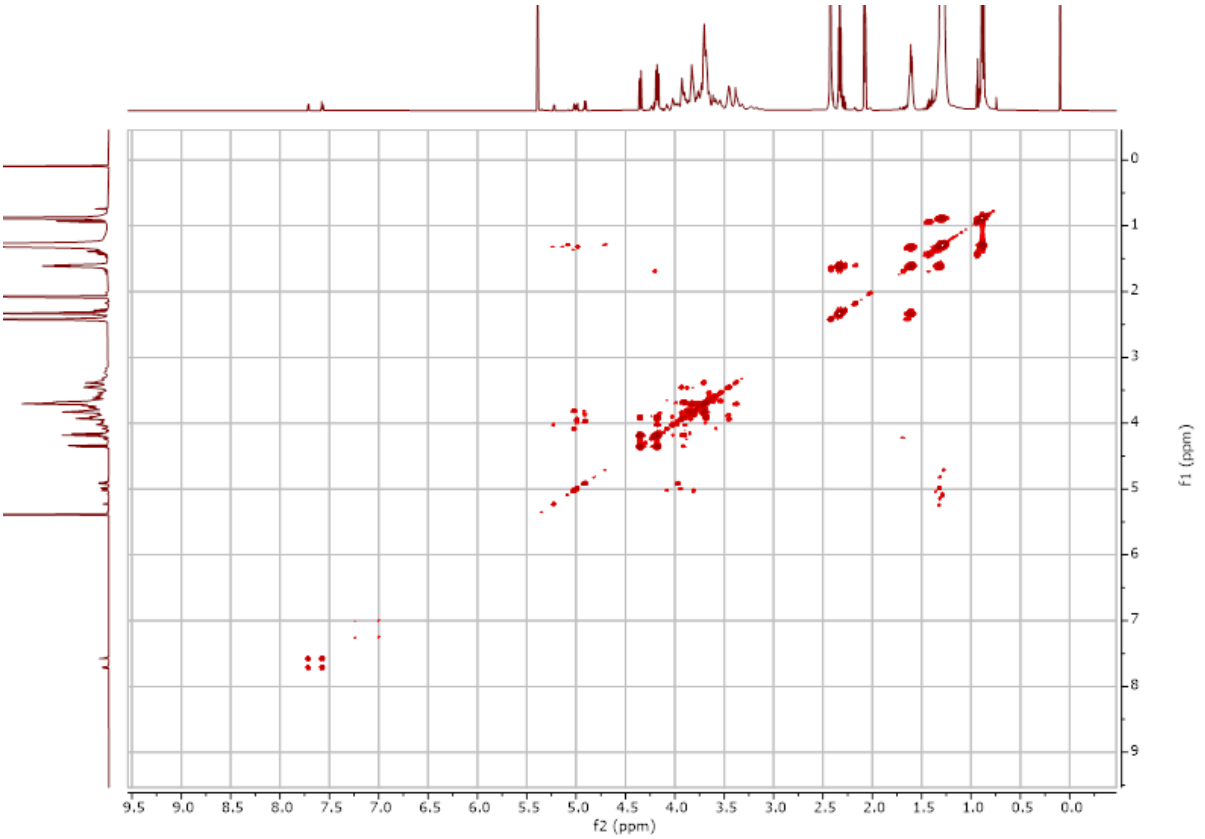


Figure A4. 2D NMR experiment: ^{1}H-^{1}H COSY of SL.

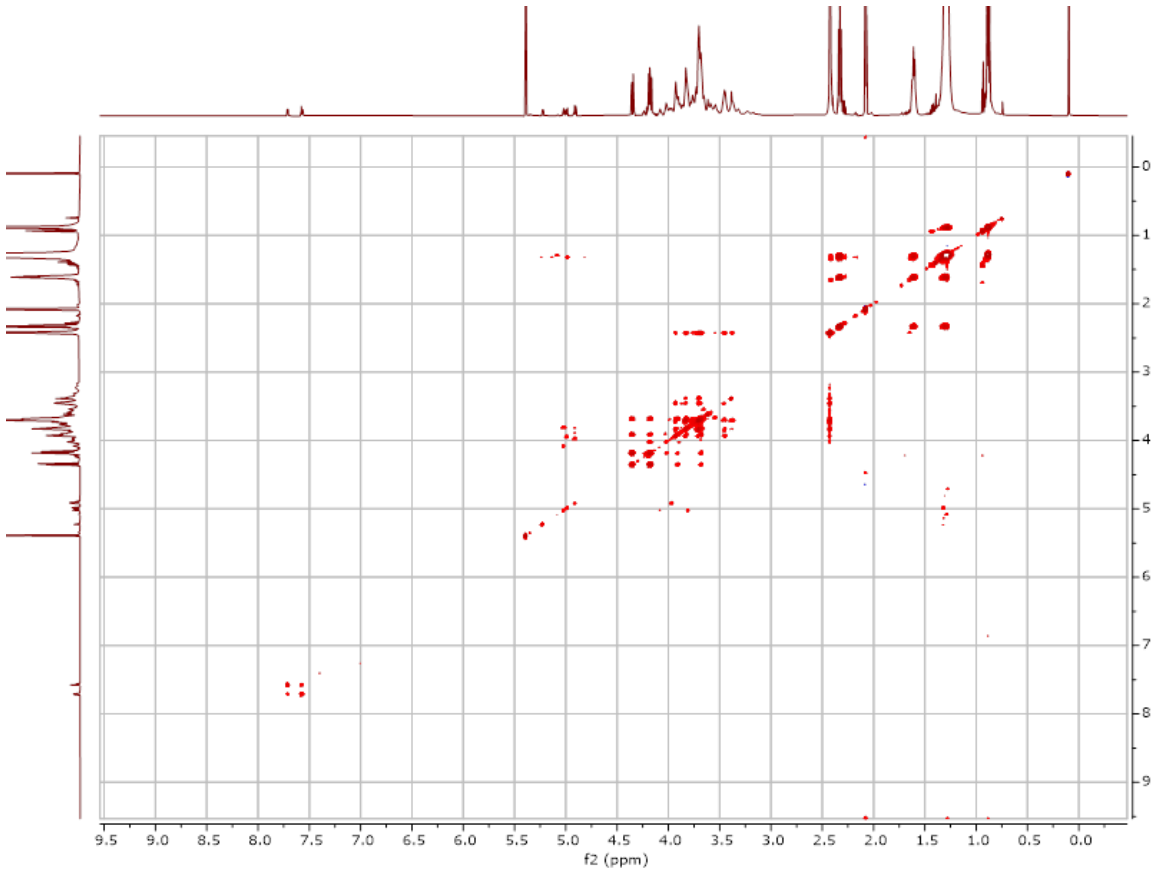


Figure A5. 2D-NMR experiment: ^{1}H-^{1}H TOCSY of SL.

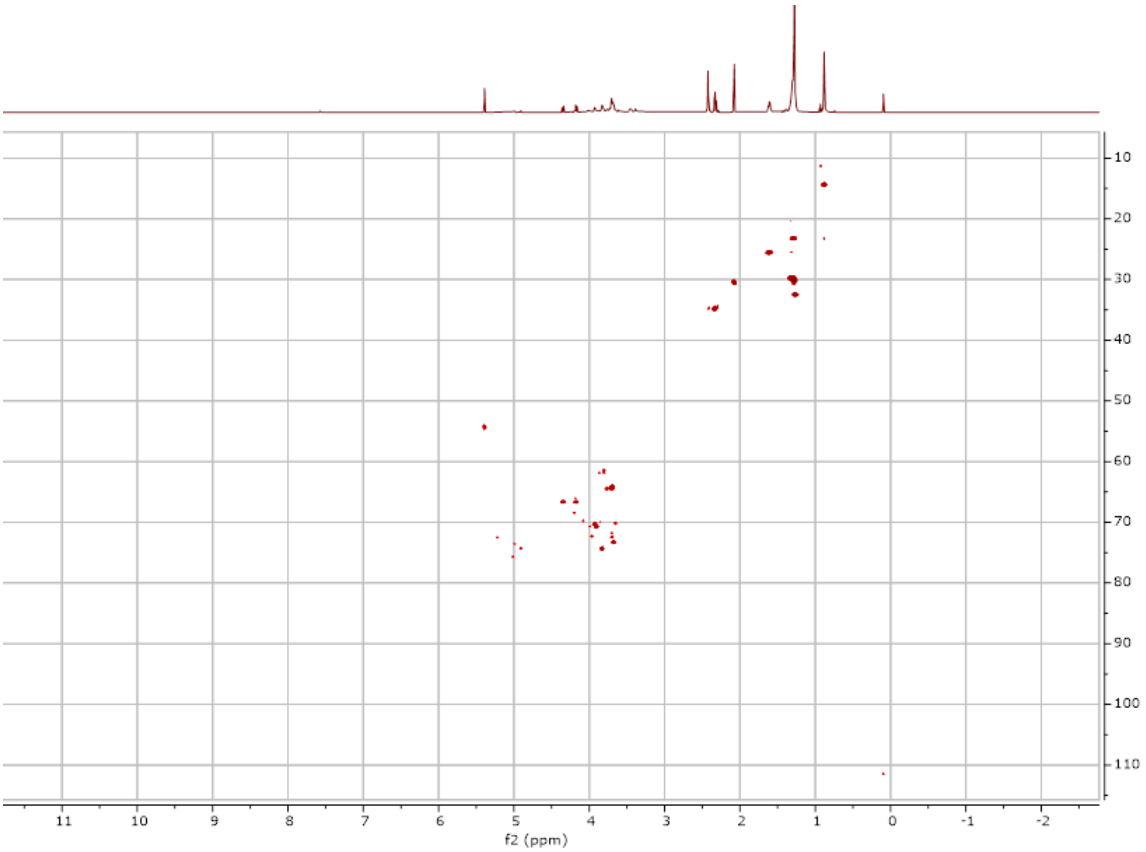


Figure A6. 2D-NMR experiment: ^{1}H-^{13}C HSQC of SL.

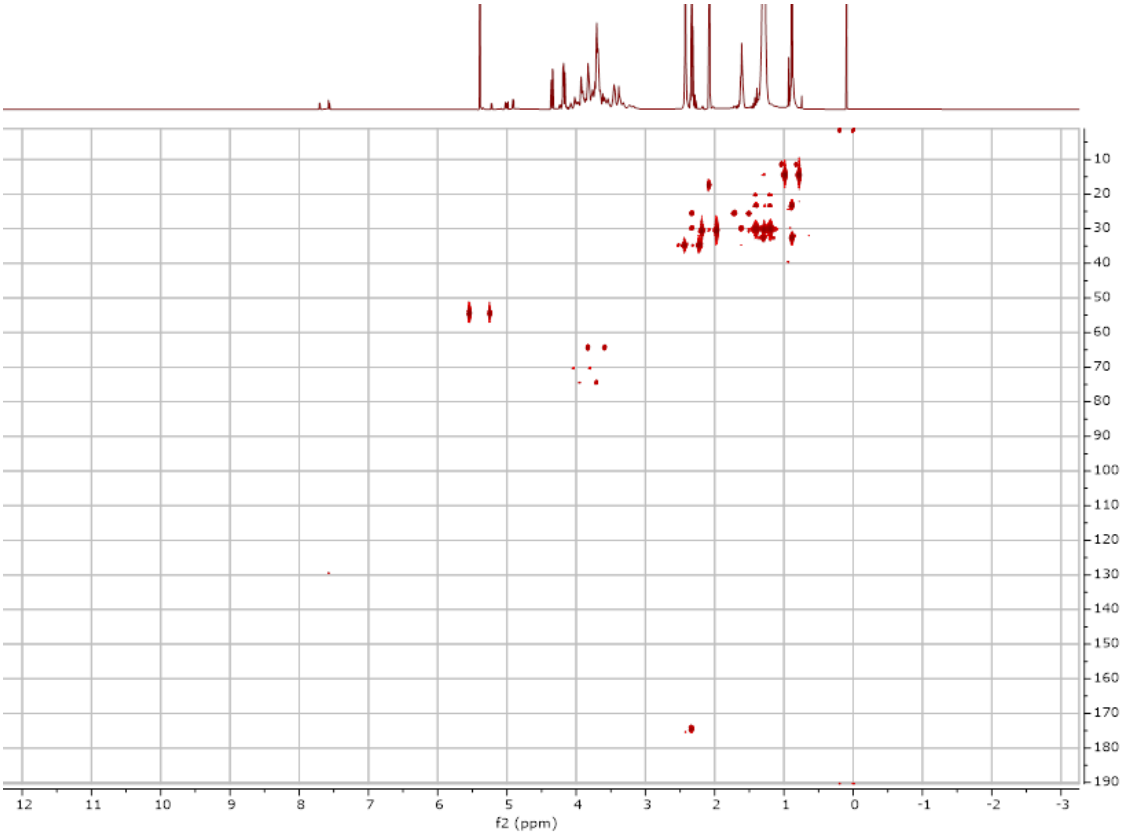


Figure A7. 2D-NMR experiment: ^{1}H-^{13}C HMBC of SL.

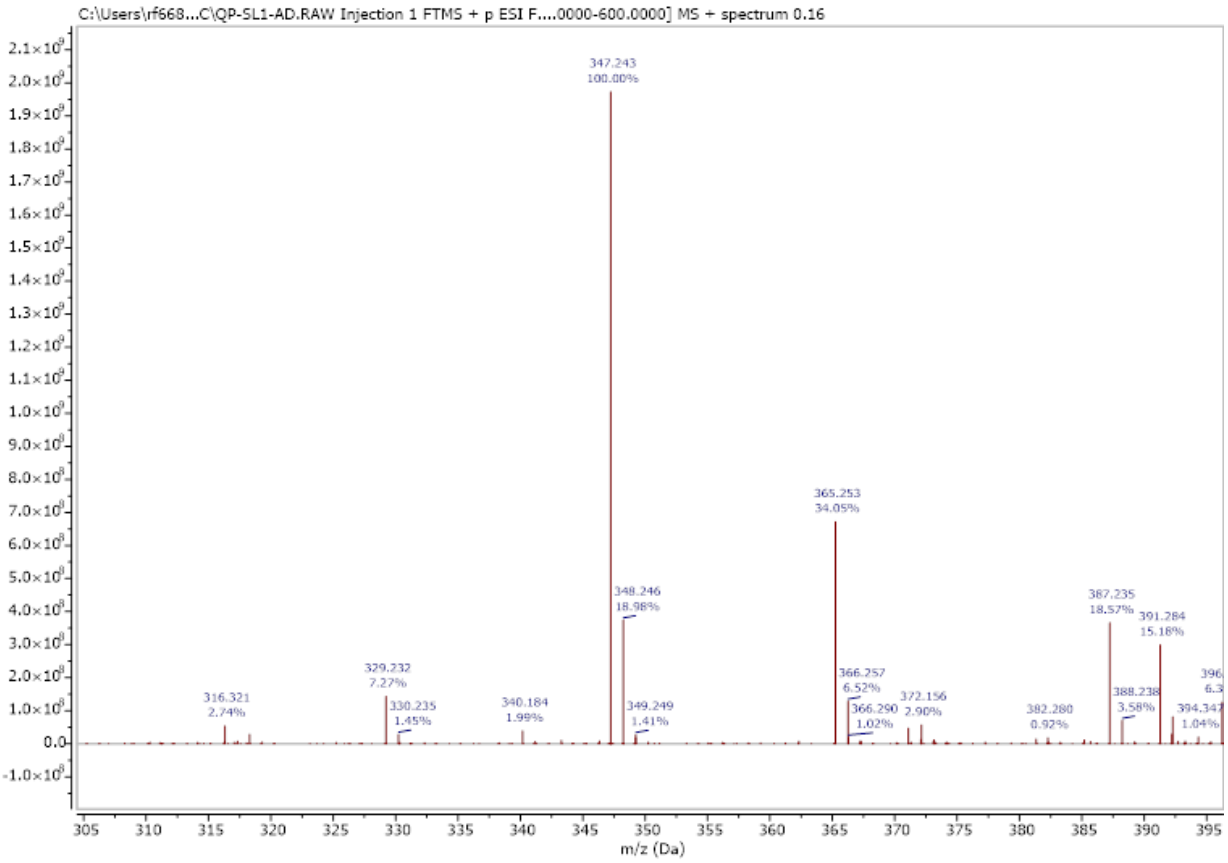
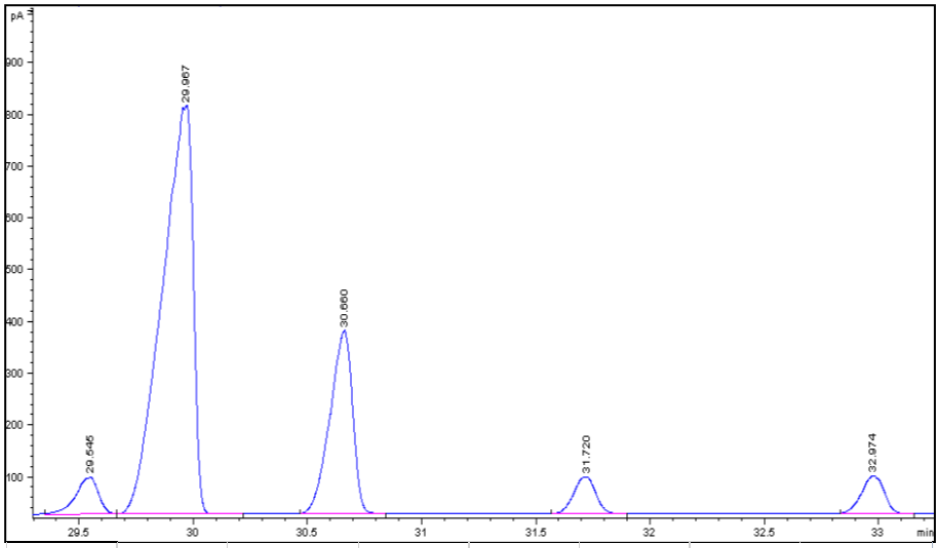


Figure A8. Mass spectroscopy using ESI-Q measurements: spectra of the purified sorbitol-6-O-laurate.



Component	Area [pA*s]	RetTime [min]	Percent	C [mg/ml]	C-Atoms	Molecular Weight [g/mol]
Methyl tetradec	132,268814	22,49713898	0,92%	0,0919051	C14:0	242,4
Methyl palmitat	567,461609	25,22681236	3,94%	0,3942926	C16:0	270,45
Methyl octadeca	352,501007	29,54556371	2,45%	0,2449303	C18:0	298,5
cis-9-Oleic acid n	6490,54932	29,96778099	45,10%	4,5098657	C18:1	296,49
Methyl Linoleate	2108,30493	29,02908325	14,65%	1,4649256	C18:2	294,47
Methyl Arachida	402,426697	29,97756767	2,80%	0,2796205	C20:0	326,56
Methyl Linolenat	442,05304	30,66054758	3,07%	0,3071543	C18:3	292,46
Methyl docosanc	393,831879	31,72079568	2,74%	0,2736485	C22:0	354,61
Methyl Erucate (	3022,94409	32,97488187	21,00%	2,1004496	C22:1	352,59
Methyl lignocera	479,548981	41,64985275	3,33%	0,3332078	C24:0	382,66
Totals	**14391,8904**					

Figure A9. Example of a GC-chromatogram presenting main FAMEs in the lipid mixture and their signal integration.

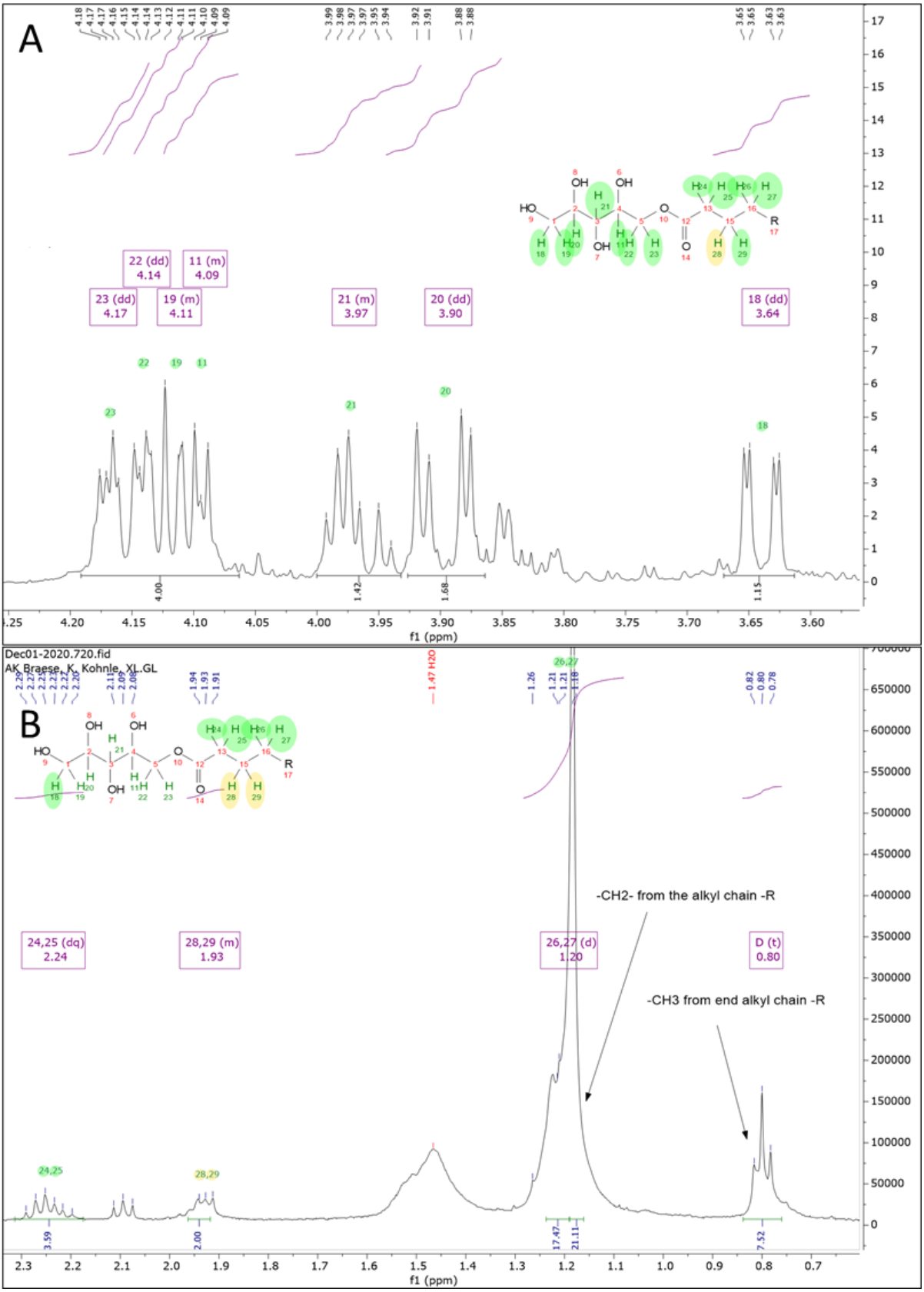


Figure A10. Treated ^{1}H-NMR experiment with zooms on the chemical shifts corresponding to the xylitol (A) and the fatty acids (B).

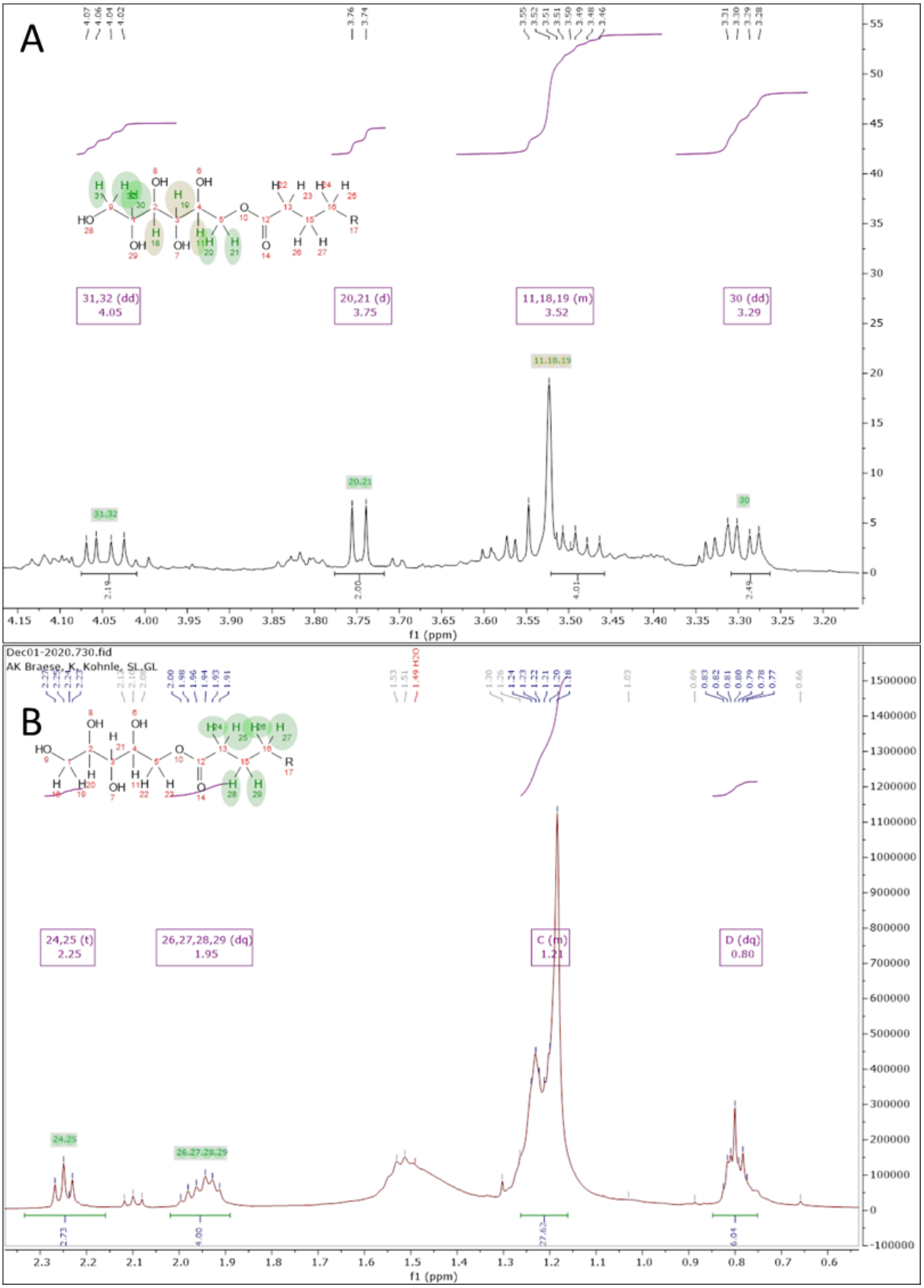


Figure A11. Treated ^{1}H-NMR experiment with zooms on the chemical shifts corresponding to the sorbitol (A) and the fatty acids (B).

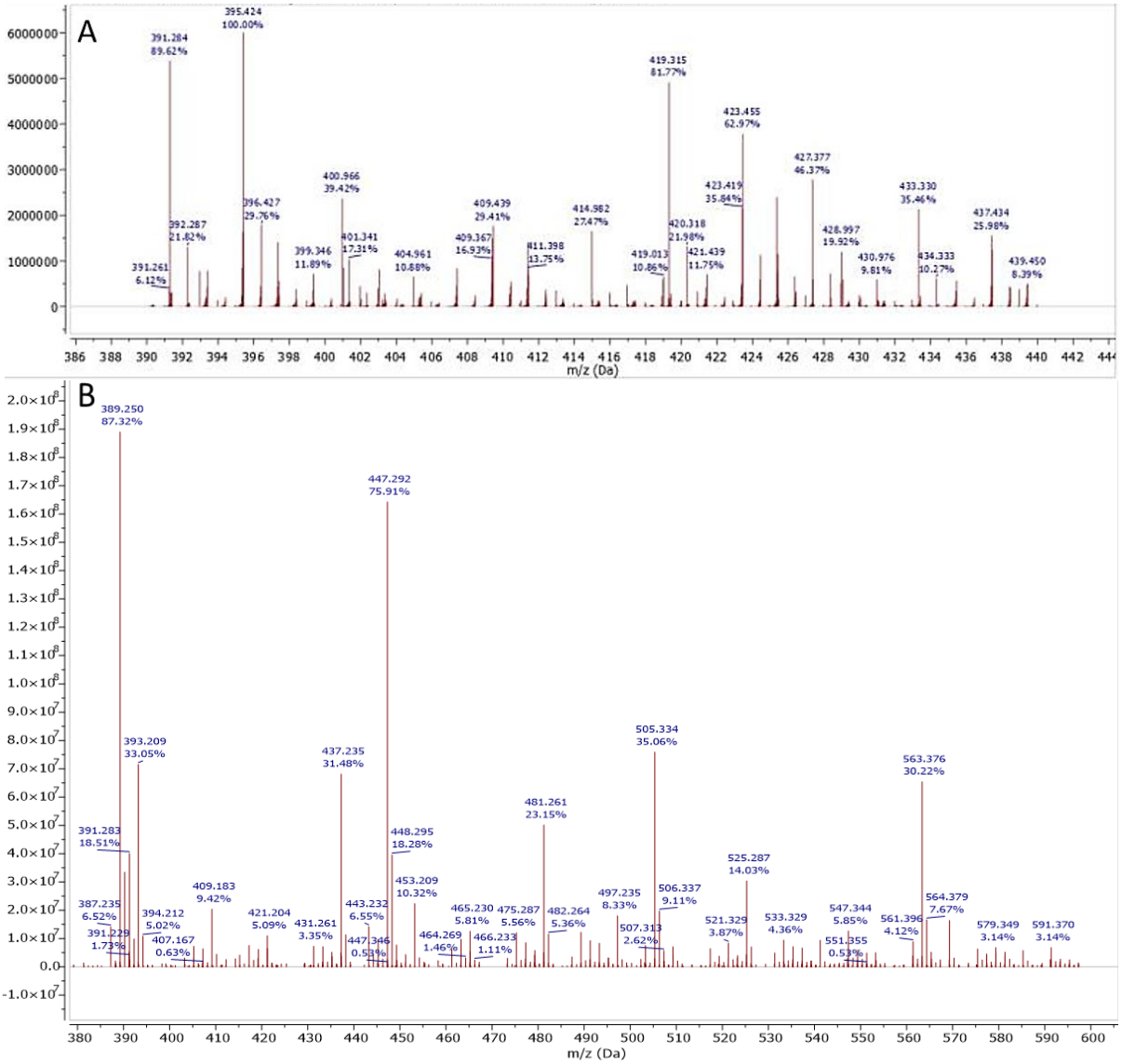


Figure A12. ESI-Q measurements of the isolated glycolipid fractions with xylitol-based esters (A) and sorbitol-based esters (B).

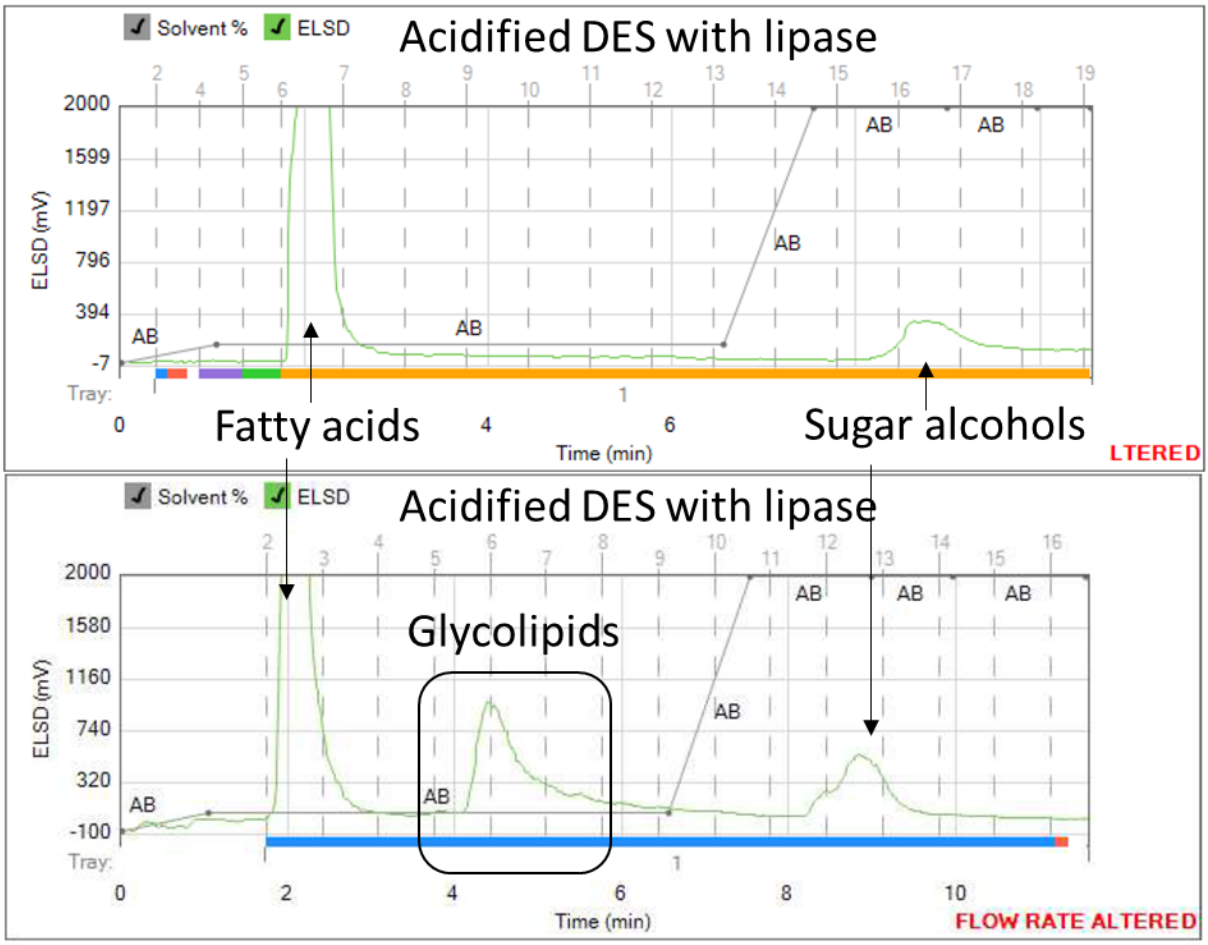


Figure A13. Chromatograms of flash chromatography purification for a standard and acidified sugar alcohol-based DES with lipase.