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CBM 13

Carbohydrate Bioengineering Meeting

May 19-22, 2019

Toulouse / FRANCE

Program book

CBM13

CARBOHYDRATE BIOENGINEERING MEETING

Program & Abstracts

May 19th to 22th, 2019

Toulouse, France

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TABLE OF CONTENTS

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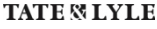	**Tate & Lyle** Tate & Lyle is a global leading provider of sweetening, enrichment, texturising and stabilising solutions and ingredients for the food and beverage market. Inspired by our purpose of Improving Lives for Generations, we work with customers to deliver great tasting, high quality and nutritious products which are enjoyed by millions of people every day. • *www.tateandlyle.com*

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SUNDAY, MAY 19TH, 2019

14:00 16:00		Registration and welcome coffee	
16:00 16:10		Welcome	
16:10 17:10		Opening lecture - **Laura Kiessling** › Glycans as microbial IDs Chair: *Magali Remaud-Siméon*	p.32
17:10 17:40		Coffee break	
17:40 19:00	SESSION 1 : Carbohydrate structure & complex carbohydrate-based matrices Chair: *Birte Svensson*		
17:40 18:20		***Jesús Jiménez-Barbero*** › Breaking the limits in understanding glycan recognition by NMR	p.33
18:20 18:40		*Stefanie Barbirz* › Host interactions of O-antigen specific bacteriophages: Linking tailspike glycan recognition to particle opening	p.34
18:40 19:00		*Wade Abbott* › Single-cell visualization of glycan uptake and strain-specific saccharolytic fingerprinting of rumen bacteria	p.35
19:00 20:00		**Poster pitch talks**	Poster number
		- *Amani Chalak* - Influence of the carbohydrate binding module on the activity of an AA9 lytic polysaccharide monooxygenase	2 p.73
		- *Jolanda M. van Munster* - Surface analysis tools identify how fungus Aspergillus niger modifies lignocellulose	4 p.75
		- *Joan Coines* - Oxazoline or oxazolinium ion? The reaction mechanism of GH18 chitinases	36 p.108
		- *Marie Sofie Moeller* - Structural determinants of GH13 alpha-glucan debranching activity and its natural endogenous regulation	46 p.118
		- *Annika Borg* - Crystallization, characterization and mechanistic analysis of a novel UDP-glucuronic acid 4-epimerase	33 p.105

		- *Federica De Lise* - RHA-P: Structural and functional insight into a nov-el bacterial α-L-rhamnosidase from Novosphingobium sp. PP1Y	39	p.111
		- *Fiona Cuskin* - α-1,6 mannosidase generates N-glycan specificity through requirement for GlcNac at the +2 subsite	37	p.109
		- *Julie Vanderstraeten* - VersaTile: A high-throughput DNA assembly method for the rapid construction and evaluation of cellulosome components	80	p.152
		- *Kristýna Slámová* - Transglycosylation activity of glycosynthase-type mutants of β-N-acetylhexosaminidase from Talaromyces flavus	78	p.150
		- *Samuel Butler* - Enzymatic synthesis of functionalised β-mannosyl conjugates from renewable hemicellulosic glycans	112	p.184
		- *Gleb Novikov* - Computational strategy for protein design based on structure-dynamics-activity relationship insights: GH11 xylanases as a case study	75	p.147
		- *Laurent Legentil* - Diversion of the arabinofuranosidase CtAraf51 for the anomeric acylation of L-arabinofuranose	95	p.167
		- *Cédric Montanier* - Immobilized enzymes at work: when surface density matters	126	p.198
		- *Elizabeth Ficko-Blean* - Carrageenan catabolism is encoded by a complex regulon in marine heterotrophic bacteria	11	p.83
20:00		Welcome reception		

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MONDAY, MAY 20TH, 2019

08:30 10:10	SESSION 2: Enzymes for carbohydrate synthesis and modification Chair: *Antoni Planas*			
08:30 09:10		***Eva Nordberg Karlsson*** › Synthesis by CAZymes from extremophiles -		p.36
09:10 09:30		*Lothar Elling* › A novel enzyme module system for the one-pot synthesis of hyaluronic acid from sucrose and N-acetylglucosamine		p.37
09:30 09:50		*Gregor Tegl* › Single step S-GlcNAcylation of peptides and proteins using a mutant hexosaminidase-		p.38
09:50 10:10		*Jiao Zhao* › Molecular study of hydrolysis/transglycosylation modulation in retaining glycoside hydrolases		p.39
10:10 10:40		Coffee break		
10:40 11:20		SESSION 2: Enzymes for carbohydrate synthesis and modification Chair: *Vladimir Kren*		
10:40 11:00		*Mahima Sharma* › Dissection of sulfoglycolytic (sulfo-EMP) pathway		p.40
11:00 11:20		*Moulis Claire* › Enlarging the toolbox of GH70 sucrose-active enzymes by mixing discovery and rational engineering		P.41
11:20 12:20		**Poster pitch talks**	Poster Number	
		- *Zhong-Peng Guo* - Developing cellulolytic *Yarrowia lipolytica* as a plat-form for the production of valuable products in consolidated bioprocessing of cellulose	120	p.192
		- *Zhi Wang* - Functional characterization of a FOS transporter sys-tem from an uncultured human gut Dorea species	19	p.91
		- *Scott Mazurkewich* - Understanding enzyme-substrate interactions in Carbohydrate Esterase family 15	106	p.178
		- *Camille F. Chastel* - A novel family of LPMO acting on cellulose identified in the secretomes of *Aspergillus* spp.	103	p.175

		- *Maria João Maurício da Fonseca* - High-throughput substrate specificity analysis of metagenomic-derived arabinoxylan-active enzymes	26	98
		- *Nicholas Lanz* - Role of the KDO Glycosyltransferase KpsS in the Biosynthesis of the Polysialyltransferase Acceptor for *Escherichia coli* K1	124	p.196
		- *Sanaullah Khan* - Identification and structural analysis of alginate oligosaccharide binding sites on β-lactoglobulin	122	p.194
		- *Constantin Ruprecht* - Generation of a glycodiversification platform for small molecules in *Escherichia coli* K12	130	p.202
		- *Sarah Desmons* - Stereocontrolled chemo-enzymatic conversion of CO_2	87	p.159
		- *Nobukiyo Tanaka* - A fungal endo-β-1,2-glucanase with a unique reaction mechanism belongs to a new glycoside hydrolase family	67	p.139
		- *Eva Madland* - The NMR structure of carbohydrate binding module 14 from human chitotriosidase and its interaction with chitin	53	p.125
		- *Manon Molina* - Exploration of the molecular determinants involved in alternansucrase specificity and polymerization	57	p.129
		- *Magda Faijes* - Metabolic engineering strategies for glycolipids production	115	p.187
		- *Tjaard Pijning* - Structural insights in starch conversion by GtfB glucanotransferase enzymes from *Lactobacilli*	60	p.132
12:20 13:50		Lunch (1h30)		
13:50 15:30		SESSION 3: Glycobiotechnologies Chair: *Carsten Andersen*		
13:50 14:30		***Emma Master*** › Biocatalytic cascades to bifunctional carbohydrates		p.42

14:30 14:50		*Simon Ladevèze* › Ultra-high-throughput droplet microfluidics CAZymes functional screening using coupled enzymatic assays	p.43
14:50 15:10		*Xuefeng LU* › Cyanobacterial Conversion of CO_2 to Sugars	p.44
15:10 15:30		*Ryo Kakutani* › Enzymatic synthesis of Glucan Dendrimer (GD) and its application for drug delivery carriers	p.45
15:30 16:30		Poster session #1	
16:30 17:00		Coffee break	

17:00 18:40	SESSION 4: Omics and CAZyme discovery Chair: *Gideon J. Davies*		
17:00 17:40		***Bernard Henrissat*** › Estimating glycan natural diversity using CAZymes	p.46
17:40 18:00		*Marie Couturier* › Large-scale screening of activities in unexplored CAZy sub-families, and distant or non-classified CAZymes	p.47
18:00 18:20		*Gabrielle Potocki-Véronese* › Ultra-high-throughput discovery of dietary and host glycan utilization pathways in gut microbiomes	p.48
18:20 18:40		*Peter Rahfeld* › Discovery of new enzymes for universal donor blood production	p.49

TUESDAY, MAY 21TH, 2019

08:30 10:10	SESSION 5: Auxiliary activities enzymes & carbohydrate esterases Chair: *Bernd Nidetzky*		
08:30 09:10		***Antoni Planas*** › Peptidoglycan deacetylases. Dual N-acetylglucosamine and N-acetyl-muramic acid specificities, structures and biological functions	p.50
09:10 09:30		*Gaston Courtade* › Structural and functional insights into the mode of action of a modular lytic polysaccharide monooxygenase	p.51
09:30 09:50		*Martin Pfeiffer* › The human GDP-mannose-4,6-dehydratase reveals the minimal active site needed for NDP-sugar dehydration	p.52
09:50 10:10		*Jane Agger* › Enzymatic cleavage of lignin-carbohydrate complexes by fungal glucuronoyl esterases	p.53
10:10 10:40		Coffee break	
10:40 12:20	SESSION 6: Glycobiotechnologies Chair: *Takashi Kuriki*		
10:40 11:20		***Barbara Ann Halkier*** › Engineering of the production of health-promoting glucos-inolates in heterologous hosts	p.54
11:20 11:40		*Paul DeAngelis* › Chemoenzymatic synthesis of thio-linked heparinoid polysaccharides for anti-cancer applications	p.55
11:40 12:00		*Vladimir Kren* › Glycosidase-catalyzed synthesis of glycosyl esters and phenolic glycosides	p.56
12:00 12:20		*Gale Wichmann* › Metabolic and enzyme engineering to produce high quantities of the steviol glycoside Rebaudioside M in S. cerevisiae	p.57
12:20 13:50		Lunch (1h30)	
13:50 15:30	SESSION 7: CAZyme engineering and computer-assisted design Chair: *Pedro M. Coutinho*		
13:50 14:30		***Narcis Fernandez-Fuentes*** › Tools for structural bioinformatics; design of a chimeric GH10 CBM-containing hydrolase	p.58

14:30 14:50		*David Teze* › Efficient conversion of GHs into transglycosylases: a conservation-based approach	p.59
14:50 15:10		*Xevi Biarnés* › BindScan: a computer-assisted protein design algorithm with applications in re-engineering CAZymes	p.60
15:10 15:30		*Mounir Benkoulouche* › Enzymatic glucosylation of a chemically-protected tetrasaccharide to access antigenic oligosaccharides	p.61
15:30 16:30		Poster session #2	
16:30 17:00		Coffee break	
17:00 18:40	SESSION 8: Polysaccharide metabolism & carbohydrate transport (Amphitheater) Chair: *Marco Moracci*		
17:00 17:40		***Mirjam Czjzek*** › Completing the pathways of polysaccharide metabolism by marine heterotrophic bacteria	p.62
17:40 18:00		*Harry Brumer* › Prevotella β-mannan utilization Loci: from the rumen to the human	p.63
18:00 18:20		*Nicole Koropatkin* › The Ruminococcus bromii amylosome: Structure of the Amy12 pullulanase and its starch-binding protein partner Doc20	p.64
18:20 18:40		*Lucy Crouch* › Novel insights into mucin degradation by key members of the human gut microbiota	p.65
20:00 23:30		Gala Dinner  Address: Espaces Vanel 1 Allée Jacques Chaban-Delmas 31500 Toulouse espacesvanel.com Subway : Marengo SNCF	

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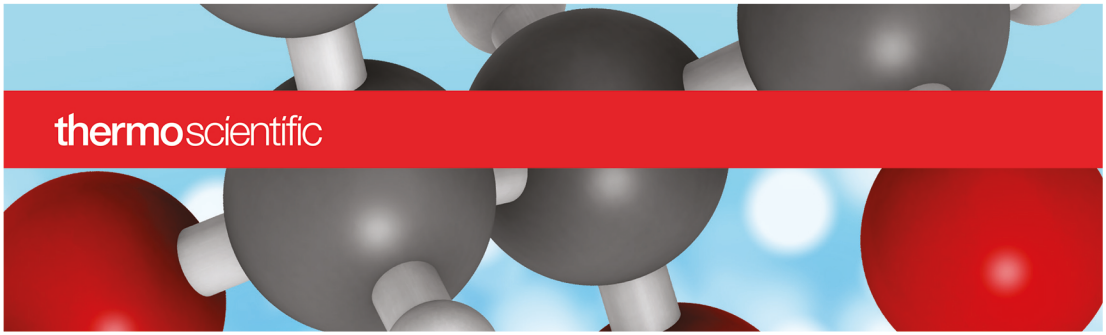
WEDNESDAY, MAY 22TH, 2019

09:00 11:30	SESSION 9: Mechanisms, structure-function relationships and dynamics of CAZymes Chair: *Steve G. Withers*		
09:00 09:40		***Shinya Fushinobu*** › Structural analysis of β-L-arabinofuranosidases in GH127 and GH146	p.66
09:40 10:00		*Carme Rovira* › Early stages of glycogen biosynthesis: mechanism of action of glycogenin	p.67
10 :00 10 :20		*Anthony Clarke* › Structural and mechanistic basis for peptidoglycan O-acetylation in both Gram-positive and negative pathogens	p.68
10:20 10:40		*Masahiro Nakajima* › Findings of new CAZymes, β-1,2-glucan-associated enzymes	p.69
10:40 11:10		Coffee break	
11:10 11:30		*Emil G P Stender* › Discovering and characterizing alginate lyases from human gut microbiota	p.70
11:30 12:30		Closing lecture - **Jean-Guy Berrin** Fungal biodiversity: a tremendous resource for CAZymes Discovery Chair: *Magali Remaud-Siméon*	p.71



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POSTER PROGRAM

Carbohydrate structure & complex carbohydrate-based matrices	page	Poster #
David Blay Improved profiling of sialylated N-linked glycans by HPAE-PAD	p.73	1
Amani Chalak Influence of the carbohydrate binding module on the activity of an AA9 lytic polysaccharide monooxygenase - **Pitch talk on Sunday, May 19th**	p.74	2
Tobias Irmscher Structure and function of exopolysaccharide modifying enzymes in pantoea stewartii biofilm	p.75	3
Jolanda M. van Munster Surface analysis tools identify how fungus Aspergillus niger modifies lignocellulose - **Pitch talk on Sunday, May 19th**	p.76	4
Mikkel Madsen Mapping the interaction site(s) of alginate oligosaccharides on β-lactoglobulin	p.77	5
Fatima Nehal Lactococcus lactis strain F-mou exopolysaccharide: High-level production, characterization, and antimicrobial activity	p.78	6
Anna Orlova Structure of solutions of carbohydrates and their derivatives, and their reactivity in glycosylation	p.79	7
Aya Zoghlami Investigation of the dynamics of lignocellulosic biomass enzymatic hydrolysis in 4D	p.80	8

Polysaccharide metabolism & carbohydrate transport	page	Poster #
Nicola Curci Xyloglucan degradation by carbohydrate active enzymes from the hyperthermophilic archaeon Saccharolobus solfataricus	p.81	9

ORAL ABSTRACTS

Glycans as Microbial IDs

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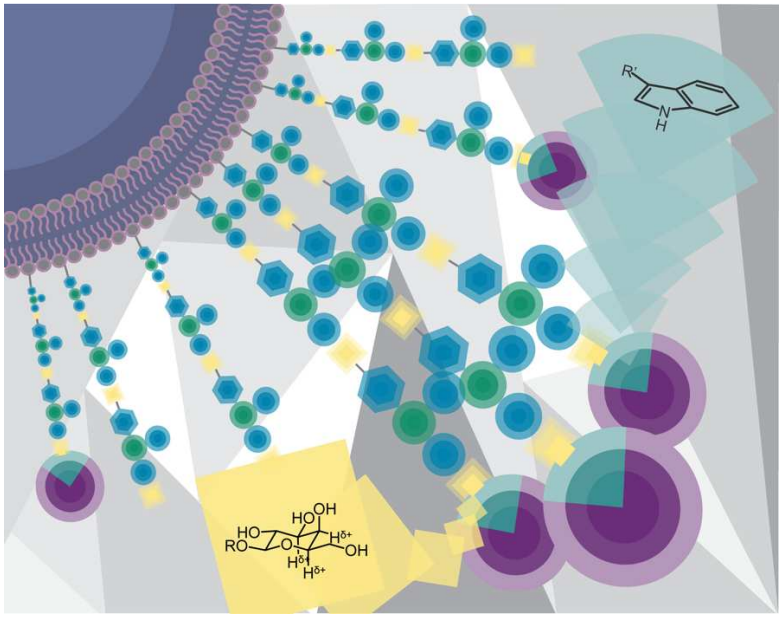
Keywords: microbiome, glycans, lectins, proteins.



Our health depends on maintaining a functional microbiome while avoiding the propagation of pathogenic microbes. Our group seeks to understand the mechanisms of microbial control by focusing on a prominent feature of the cell's exterior—the carbohydrate coat. From humans to fungi to bacteria, all cells on Earth possess a carbohydrate coat. A critical role of this coat is to serve as an identification card. Our group has been examining the role of carbohydrate-binding proteins, lectins, in influencing our microbiota and in immune defense. This seminar will focus on understanding the basis of carbohydrate-protein interactions and how they are used to control microbes. We envision that our findings can lead to alternative means to combat pathogens, methods for rapid approaches to ID microbiota, and the development of new strategies to regulate microbiome composition to promote human health.

Breaking the limits in understanding glycan recognition by NMR

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Keywords: NMR, Molecular Recognition, Glycans, Lectins.

Molecular recognition by specific targets is at the heart of the life processes. In recent years, it has been shown that the interactions between proteins (lectins, enzymes, antibodies) and carbohydrates mediate a broad range of biological activities, from fertilization and tissue maturation, to pathological processes. The elucidation of the mechanisms that govern how sugars are accommodated in the binding sites of these receptors is currently a topic of interest. Thus, unravelling the structural and conformational factors and the physicochemical features that rule the interactions of these molecules is of paramount interest.

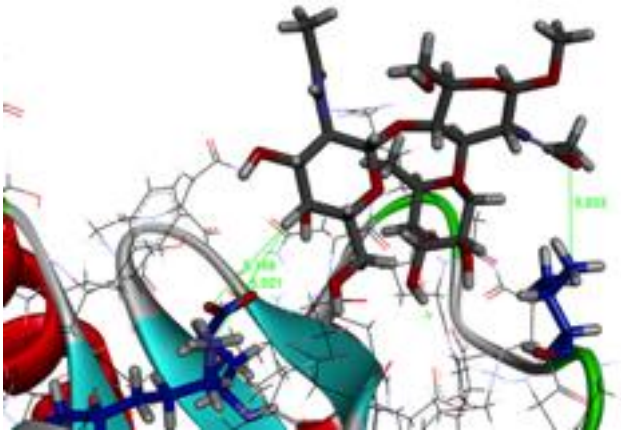

Figure 1. NMR view of the recognition of one of the histo blood group antigens by a C-type lectin

This presentation is focused on the application of state-of-the-art NMR methods both from the ligand and receptor's perspective to study molecular recognition processes between receptors of biomedical interest and glycans. Lectins, antibodies, and enzymes, both wild type and mutants, have been used as receptors with the final aim to know and to evaluate the relative importance of polar (hydrogen bonding, electrostatic interactions) and non-polar (van der Waals, CH-π) forces in the recognition process. As recent examples, key details of glycan recognition by C-type lectins, galectins, and hemagglutinins will be shown, with special emphasis in the application of novel ^{19}F and paramagnetic-NMR methods [1-5].

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Host interactions of O-antigen specific bacteriophages: Linking tailspike glycan recognition to particle opening

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Keywords: bacteriophage tailspike, O-antigen, lipopolysaccharide, glycan hydrolase

The polysaccharide O-antigen layer that covers Gram-negative bacterial surfaces is a protective barrier against bacteriophage infection. In turn, bacteriophages exploit the O-antigen as a receptor essential for infection, *i.e.* they exclusively grow on smooth strains [1]. These phages carry tailspike proteins with glycoside hydrolase activity needed to position the particle on the outer membrane [2]. We present *in vitro* studies of glycan-triggered DNA ejection of different *E. coli* and *Salmonella* phages. [3, 4]. Here, lipopolysaccharide is sufficient for particle opening and DNA release *in vitro.* We found different kinetics of *in vitro* DNA ejection depending on the morphology of the bacteriophage tail. Our experiments define the minimal bacterial glycan receptor structures that can initiate particle opening *in vitro*. They provide an important link between bacteriophage structural rearrangements in the tail and the subsequent steps for membrane penetration *in vivo.*

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Single-cell visualization of glycan uptake and strain-specific saccharolytic fingerprinting of rumen bacteria

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Keywords: glycan metabolism, rumen bacteria, fluorescent glycan conjugate, super resolution confocal microscopy

Carbohydrates that comprise the cell wall of plants and fungi represent underexploited opportunities to selectively modulate rumen microbiomes with the goal of benefiting animal production. Chemically distinctive polysaccharides (e.g. yeast mannan), added to the diet, have the potential to stimulate dynamic changes in community structure, and improve feed efficiency and host performance. Commonly, metabolic responses of microorganisms to dietary polysaccharides are studied indirectly, primarily by sequencing methods, which has created a bottleneck in translating sequence information into function. In this regard, molecular tools and "direct" methods that help unravel the mechanisms driving polysaccharide-microbe interactions in higher-throughput will benefit how we interpret and program functional changes in microbiome communities. To address this, our team has been deploying a multifaceted approach, involving selective anaerobic isolation, comparative metabolic pathway and saccharolytic fingerprinting analyses [1], RNA-sequencing, single-cell super resolution structured illumination microscopy of fluorescent carbohydrate uptake [2], and streamlined enzymology, to evaluate saccharolytic responses of intestinal bacteria and microbiomes to dietary polysaccharides. Presented here is high-resolution *in silico* analyses, X-ray crystallography, selective growth profiling, and single-cell fluorescence microscopy to demonstrate that strains of rumen *Bacteroides* spp. have evolved tailored pathways for the competitive utilization of yeast mannan in the cattle rumen ecosystem. This research platform can be extended to study other glycan-microbiome relationships and presents a useful combination of molecular tools for carbohydrate active enzyme discovery.

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Synthesis by CAZymes from extremophiles.

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Keywords: Glycoside hydrolase, GH1, GH13, alkyl glycoside, surfactant

Enzyme properties are a significant factor for transglycosylation in glycoside hydrolases (GH). In retaining enzymes, the catalytic mechanism involves a glycosyl-enzyme intermediate, which can be deglycosylated either by water or by another acceptor molecule, yielding hydrolysis products or a glycoconjugate, respectively. The yield is determined by the acceptor specificity of the enzyme, often quantified as the ratio of transferase over hydrolase activity.

We have focused on the production of alkylglycosides, in transfer reactions. Alkyl glycosides are surfactants, and constitute one of the most widely used commodities in the world. They are important ingredients in a wide variety of products, such as laundry detergents, paint, food, pharmaceutical formulations and many others. Surfactants have also over time caused large environmental problems, due to poor biodegradability and toxicity, hormone disruption and other harmful effects on living organisms. Compared to many other surfactants used, the alkyl glycosides are an environmental friendly alternative.

Enzymatic synthesis of alkyl glycosides using glycoside hydrolases is well studied, but has yet to reach industrial scale, primarily due to limited yields. Several previous studies have aimed at increasing alkyl glycoside yield by improving the ratio between transferase over hydrolase activity through protein engineering. In this area, work using enzymes candidates from GH1 will be presented. In addition, extension of the hydrophilic headgroup in alkyl glycosides is another area of interest. Oligomeric saccharide alkyl glycosides are extremely difficult to synthesize chemically, and although they can be made enzymatically, it is still a challenge to find efficient enzymes catalyzing the reaction. We have explored use of enzymes from the α-amylase family to produce this type of surfactants. The enzymes mainly originate from GH13 and results from our work will be given in this presentation.

A novel enzyme module system for the one-pot synthesis of hyaluronic acid from sucrose and *N*-acetylglucosamine

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Keywords: Hyaluronan, glycosaminoglycan, nucleotide sugars, sucrose.

Hyaluronic acid (hyaluronan, HA) is a linear polysaccharide composed of repeating disaccharide units of glucuronic acid (GlcA) and *N*-acetylglucosamine (GlcNAc), (4GlcAβ1,3GlcNAcβ1-). HA polymers with defined molecular size and low dispersity are desired for applications in biomedicine and cosmetics. Bifunctional plasma membrane-integrated (Class I) or peripheral (Class II) HA synthases (HAS) are involved in HA biosynthesis and utilize UDP-GlcA and UDP-GlcNAc as monosaccharide donors [1]. Class II *Pasteurella multocida* HAS (pmHAS) was utilized for in vitro synthesis of size-defined monodisperse HA preparations [2]. However, nucleotide sugars are expensive substrates and an issue for HA synthesis optimization.

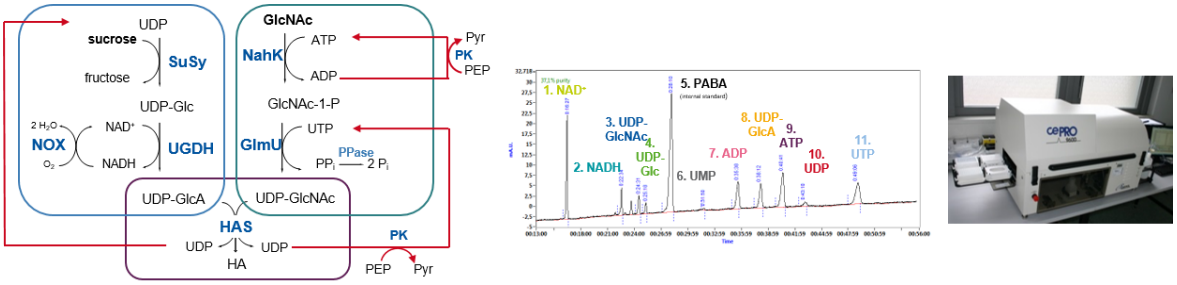

Figure 1. EMS for HA synthesis and high-through-put analysis of reaction cascades by MP-CE.

We here present a novel enzyme-module-system (EMS) for the in vitro one-pot synthesis of HA with in situ production and regeneration of nucleotide sugars starting from sucrose and GlcNAc [3] (Figure 1). Multiplexed capillary electrophoresis (MP-CE) was used for analysis and optimization of reaction cascades [4,5]. Enzymes from the commercial HA production host *Streptococcus zooepidemicus* were applied as novel enzymes for the synthesis of UDP-GlcA and UDP-GlcNAc. A comprehensive reaction analysis approach was developed for reaction optimization in 96-well microtiter plate format to obtain high-Mw HA (> 2 MDa) within a few hours. Key parameters influencing polymerization rate and Mw of HA by pmHAS were metal ion cofactors, enzyme kinetics and substrate ratio. UDP-GlcA regeneration by sucrose synthase proved as highly favorable for HA polymerization. In summary, the presented optimized EMS is an excellent starting point for cost-efficient HA synthesis from cheap and renewable starting material.

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Single step S-GlcNAcylation of peptides and proteins using a mutant hexosaminidase

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Keywords: glycoprotein synthesis, glycosynthase, thioglycoligase, hexosaminidase

Carbohydrate mimics such as thioglycosides are hydrolase-resistant derivatives of O-linked glycosides that can serve as valuable probes for studying the role of glycosides in biological processes. Traditional chemical syntheses of thioglycoside analogues of O-GlcNAc-modified peptides and proteins require lengthy, multi-step approaches.
We present the development of efficient, enzyme-mediated syntheses of thioglycosides, including S-GlcNAcylated peptides and proteins, using a mutant GH 20 hexosaminidase from *Streptomyces plicatus*, SpHex E314A. The mutation of the catalytic acid/base glutamate (E314) to an alanine resulted in a potent thioglycoligase which uses readily available GlcNAc and GalNAc donors and couples them to a remarkably diverse set of thiol acceptors.

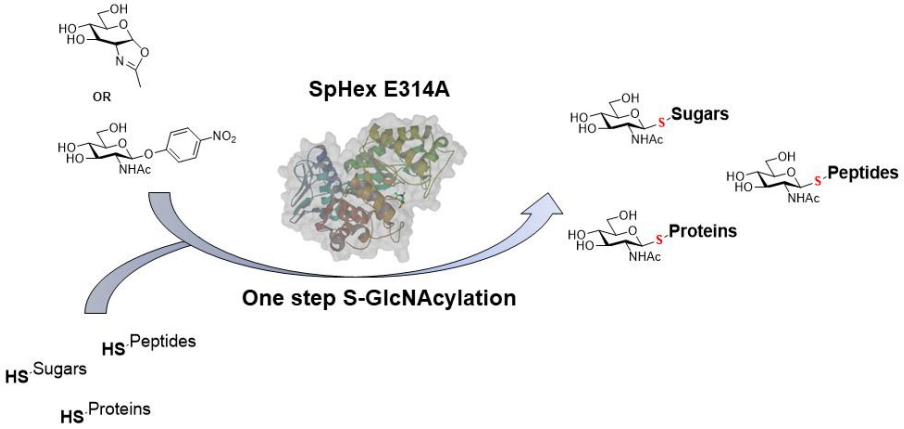

Figure 1. A GH20 thioglycoligase as a versatile catalyst for thio-glycoconjugate synthesis.

Thioglycoligation using 3-, 4-, and 6-thiosugar acceptors from a variety of sugar families produces S-linked disaccharides in nearly quantitative yields. The set of possible thiol acceptors also includes cysteine-containing peptides and proteins, rendering this mutant enzyme a promising catalyst for the production of thio analogs of biologically important GlcNAcylated peptides and proteins.[1] Further developments based on this study also be presented.

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Molecular study of hydrolysis/transglycosylation modulation in retaining glycoside hydrolases

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Keywords: retaining glycoside hydrolases, transglycosylation, molecular design, enzyme promiscuity

The chemical synthesis of glycosidic bonds, involved in the design of many molecules of interest, has often proven to be quite challenging while the vast biodiversity of retaining glycoside hydrolases (rGHs) constitutes a reservoir of readily available and robust enzymes for the catalysis of glycosynthesis reactions. Not many of these enzymes are capable of significant innate synthesis (*i.e.* via transglycosylation). This is because the transglycosylation and hydrolysis catalyzed by these enzymes are in competition. Nevertheless, some atypical rGHs can be described as transglycosylases (TGs). These catalyze transglycosylation reactions without any significant interference from hydrolysis, meaning that they perform glycosynthesis efficiently. Recent work by our group has shed light on the hydrolysis/transglycosylation (H/T) equilibrium in rGHs [1], whose activities have been evolved towards transglycosylase activity leading to the molecular design of new TGs [2,3].

Using a mixed protein engineering strategy, the hydrolytic GH51 α-L-arabinofuranosidase from *Thermobacillus xylanilyticus* (*Tx*Abf) has previously been used as a template to create transarabinofuranosylases [2]. Presently, we are pursuing this work in order to finely probe the H/T partition and better define engineering rules for the creation of TGs from rGHs. This presentation will focus on active site mapping of *Tx*Abf, and will reveal some of the kinetic data that we have collected for mutants that mainly catalyze transglycosylation reactions. These will be coupled to data from structural and molecular dynamics studies. Within this framework, we will also describe how certain mutants display greater aptitude for transglycosylation when using an alternative donor substrate, namely nitrophenyl-activated β-D-Gal*f*, instead of the original *p*NP-α-L-Ara*f*, and various acceptor substrates. Our results are important, because they not only further our knowledge of the molecular determinants that govern the H/T partition in rGHs, but also open avenues towards the creation of efficient transgalactofuranosylases. These latter might be useful for the synthesis of chemotherapeutic compounds.

Acknowledgments. J.Z. receives a PhD fellowship stipend from the China Scholarship Council (CSC).

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Dissection of Sulfoglycolytic (sulfo-EMP) pathway

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Keywords: X-ray crystallography, glycolytic enzymes, EMP pathway, sulfoquinovose.

Sulfoquinovose (SQ), a sulfonated glucose present within plant sulfolipids (SQ-diglyceride and its metabolites), is a major component of global sulfur cycle with its estimated production amounting to 10 billion tonnes annually [1]. A bacterial sulfoglycolytic pathway analogous to the classical glycolytic Embden-Meyerhof-Parnas (EMP) pathway has been reported [2]; however, structural and biochemical characterization of core enzymes involved in the degradation of sulfoquinovose is lacking. Here we present, crystal structures of sulfo-EMP enzymes, in complex with their proposed intermediates, as well as the kinetic studies to shed light on their mechanisms, the determinants of sulfo-sugar specificity and their selectivity over glycolysis intermediates. The sulfonate recognition sequences thus identified further inform our search for sulfoglycolytic enzymes in different organisms in varied environmental niches.

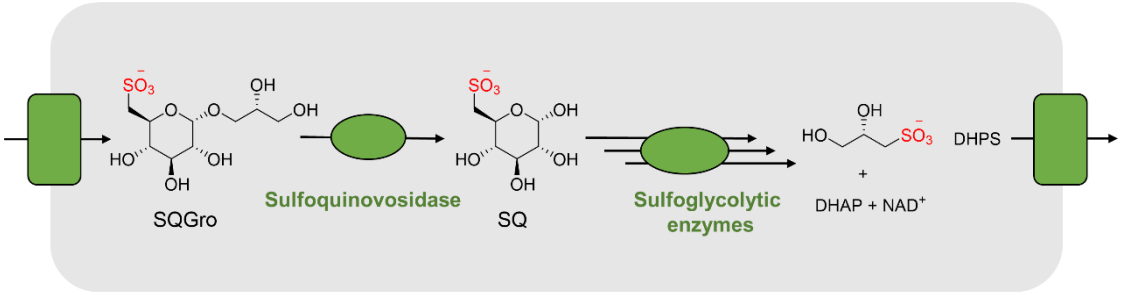

Figure 1. Summary of the sulfo-EMP pathway. SQGro, a sulfoquinovosyl metabolite of SQDG, is imported into the SQ-utilizing organisms and hydrolysed to liberate SQ [3] which is metabolized to release C3-sulfonate product 2,3-dihydroxypropane-1-sulfonate (DHPS), dihydroxyacetone phosphate (DHAP) and NAD^+.

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Enlarging the toolbox of GH70 sucrose-active enzymes by mixing discovery and rational engineering

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Keywords: GH70 α-transglucosylases, structure-function relationships, carbohydrate synthesis, glycodiversification.

Environmental and sustainability concerns push forward the research on renewable and recyclable bio-derived polymers with well-defined and highly reproducible structures in order to replace the petroleum-based products. With the progress in bioinformatics, structure-function studies, screening technologies and enzyme engineering, new enzymes are discovered and can be further tailored to produce innovative biosourced compounds. In this context, we recently filled our enzyme collection with new GH70 α-transglucosylases that catalyze the formation of a broad variety of polymers of glucosyl units (α-glucans) from sucrose.

This presentation will first focus on the structure-function relationship studies of these intriguing enzymes, distinguishable by their linkage specificity or ability to finely control the size of the produced polysaccharides. In particular, the resolution of several 3D structures -unliganded or in complex with oligosaccharides- allowed us to decipher structural features playing a key role in polymer elongation, enzyme processivity, and/or linkage specificity [1,2]. These findings open promising strategies for GH70 enzyme engineering aiming at customizing α-glucan architectures on purpose. For illustration, recent developments for the synthesis of block copolymers composed of several covalently linked α-glucans with contrasting structures and physico-chemical properties [3] will be shown, as well as the design of eco-friendly processes for the production of controlled size dextrans.

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Biocatalytic cascades to bifunctional carbohydrates

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Keywords: telechelic molecules, carbohydrate oxidoreductase, glucuronidase, transaminase.

Genomics initiatives have uncovered the critical importance of microbial enzymes to expanding the range of products that can be made from plant biomass (i.e., lignocellulose). So far, most applications of such enzymes focus on the deconstruction of lignocellulose to monosaccharides and monolignols for subsequent fermentation to fuels and target chemicals. While necessary for capturing the full potential of renewable plant biomass, this approach inevitably foregoes the value that can be achieved by realizing the inherent performance attributes of native biomass structures. In this presentation, I will describe our efforts to discover and develop enzymes and enzyme systems that introduce new chemical functionality to underused lignocellulose fractions, leading to bifunctional carbohydrates primed for polymerization or use as bio-based crosslinkers. In particular, this presentation will describe our characterization of family AA5_2 galactose oxidases [1], family AA7 glucooligosaccharide oxidases [2], accessory hemicellulases [3,4], and carbohydrate-active transaminases [5], and their combination into cell-free enzyme cascades that create bifunctional carbohydrates from diverse carbohydrate substrates. In addition to high atom economy, this approach to bio-based chemicals and materials retains valuable properties of the starting carbohydrate substrate.

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Ultra-high-throughput droplet microfluidics CAZymes functional screening using coupled enzymatic assays

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Keywords: Ultra-high throughput, droplets, natural substrates, microfluidics.

Screening campaigns strongly relies on the size of the sequence space that can be sampled, may it be for directed evolution of an enzyme or screening of metagenomic samples. One of the latest technologies of ultra-High-Throughput Screening (uHTS) and miniaturization is based on *in-vitro* compartmentalization combined with microfluidic systems that allow droplet sorting. Based on single-cell encapsulation in droplets of water-in-oil emulsion, this technique allows to find a unique functionality from millions of variants and revolutionizes both the scale and speed of screening (Fig. 1) [1]. Microchips allow ultra-high throughput picoliter-scale single cell phenotyping at a rate of over 10^6 individual enzyme reactions per hour, using less than a microliter total reagent volume [2].

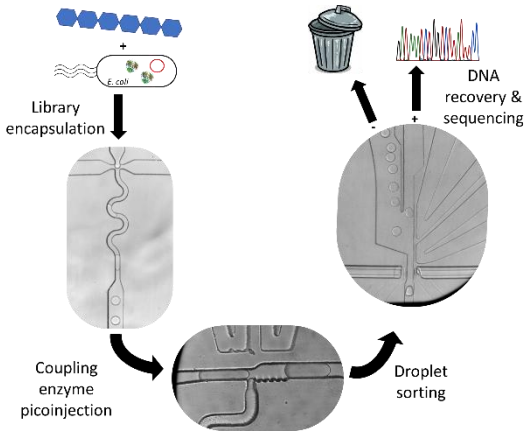

Figure 1. Schematic droplet functional screening. Library members are assayed in droplets containing the polysaccharidic substrate. The coupling enzyme is picoinjected together with lysis agent in order to allow the cascade of reactions to occur. Droplets are sorted either by absorbance or fluorescence. As the droplet compartment maintain the phenotype-genotype linkage, DNA from positive hits can be recovered.

Functional screening of Carbohydrate Active Enzymes (CAZymes) is challenging because of the optically inactive nature of the substrates and the type of reactions products that are screened for. In addition to the promiscuous nature of many CAZymes that causes high recovery of false-positive hits, typical commercially available chromogenic/fluorogenic substrates (pNP/4-Methylumbelliferyl -sugars) are unusable in droplets because the released chromophore is oil soluble and leaks out from positive droplets and cause cross-contamination. To overcome these issues, we are developing a cascade of reactions strategy in which only natural substrates are used. Accessory enzymes are used to convert the released carbohydrates generated by the 'hit' very specifically, and produce monitorable co-products, such as NADH or H_2O_2. We are currently focusing on the adaptation of such cascade of reactions to the nanodroplet scale in order to set up functional screens for polysaccharide degrading CAZymes (xylanase, glucuronidase, Ara*f*ase, etc…) that would have a broad use the context of CAZyme evolution and metagenomic screening.

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Cyanobacterial Conversion of CO_2 to Sugars

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Keywords: Cyanobacteria, Photosynthesis, Sucrose, Salt Stress.

Biorefinery technologies provide promising solutions to achieve sustainable development facing energy and environment crisis, while abundant sugar feedstock is an essential basis for biorefinery industries. Photosynthetic production of sucrose with cyanobacteria directly utilizing solar energy and carbon dioxide is an alternative sugar feedstock supply route with great potentials. Lots of cyanobacteria strains could synthesize and accumulate sucrose as osmo-protective compound to resist extracellular salts stress, however the sucrose capacities of the natural strains were quite limited for application.

Utilizing a typical cyanobacteria strain *Synechocystis* sp. PCC6803 as a model, essential genes for sucrose synthesis capacities were identified, and a combinatory strategy by enhancing the synthesis pathway and weakening the competitive pathway was developed, resulting in a 4-fold increased intracellular sucrose accumulation.

For more robust photosynthetic production of sucrose, *Synechococcus elongatus* UTEX2973, a fast-growing cyanobacteria with significant tolerances to high light and high temperature was adopted as a chassis. Introduction of an *Escherichia coli* derived permease CscB into UTEX2973 enabled efficient secretion of sucrose and significantly promoted the final titers. Combining condition optimizations, 8.7 g/L sucrose was produced by the engineered strain during a semi-continuous cultivation process for 21 days.

Scientific basis and regulatory mechanisms for the salts-stress induced sucrose synthesis in cyanobacteria was also explored. Through physiological and biochemical assays, we show that during salts stress, ionic effects, rather than osmotic effects, plays the main role for induction of sucrose synthesis in cyanobacteria. The concentrated ions directly activate the essential enzyme for sucrose synthesis and simultaneously inhibit the main degrading enzyme, resulting in sucrose synthesis and accumulation in response to salt stress.

For development of a salt-stress independent sugar production route in cyanobacteria, we then performed systematic genetic manipulations with *Synechococcus elongatus* PCC7942, another important model cyanobacterium strain, and achieved effective photosynthetic production of glucose, the most important and representative sugar feedstock for biorefinery industries.

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Enzymatic synthesis of Glucan Dendrimer (GD) and its application for drug delivery carriers

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Keywords: glycogen, dendrimer, glucan phosphorylase, drug delivery system (DDS)

Dendrimers are highly branched and star-shaped polymers with nanometer-scale dimensions. These nanostructured macromolecules are known for their defined structures, versatility in drug delivery and high functionality whose properties resemble with biomolecules.

We successfully developed the novel method to synthesize the glycogen-like polymer (glucan dendrimer; GD) from starch by using several enzymes. This glucose polymer has very attractive characteristics such as a spherical molecule, nano-sized particle, controllable molecular size, and the numerous modifiable branched chains as a dendrimer. Furthermore, it is possible to substitute glucose at non-reducing end of GD with galactose, mannose, glucosamine, glucuronic acid, or *N*-acetylglucosamine residues by using certain enzyme [1-2]. We are currently challenging to utilize GD as drug delivery carriers.

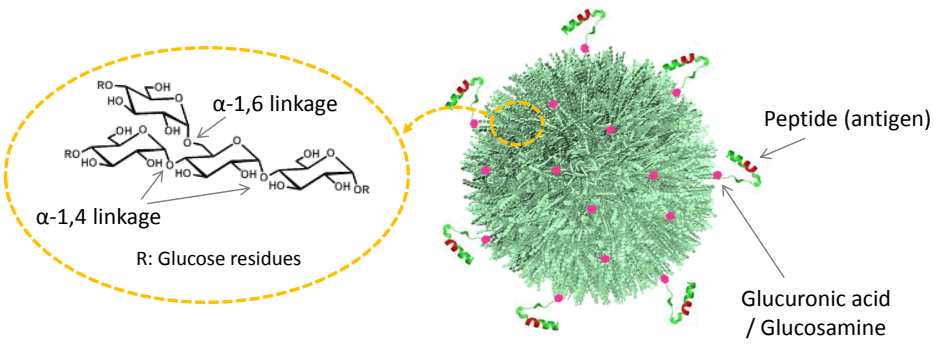

Figure 1. Illustration of GD; GD having glucosamine and/or glucuronic acid residues are especially useful since they can be conveniently conjugated with other functional molecules (peptides, nucleotides, and others). .

To investigate whether GD functions as the drug carrier, its in vivo kinetics and anti-tumor effects in mice were examined. We observed that the fluorescence-labeled GDs were tranported to lymph nodes efficiently. When GDs were conjugated with ovalbumin (OVA)-peptides, their administration suppressed tumor growth in OVA-expressed tumor-bearing mice. These findings suggest that GDs provide valuable function towards drug delivery carriers.

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Estimating glycan natural diversity using CAZymes

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Keywords: glycans, CAZymes, glycogenomics, glycobioinformatics.

Laine has calculated that $>10^{12}$ hexasaccharides built of D-hexoses are theoretically possible [1], and everyone agrees that the diversity of glycan structures is huge although there is no estimate of natural glycan diversity. Here we wished to examine glycan diversity based on real experimental biological and biochemical data. Bacteria of the Bacteroidetes phylum are found in all ecosystems across the planet (animal digestive tract, marine, soil, deserts, etc) and are utilizing natural glycan diversity for carbon acquisition. In these bacteria, carbohydrate-degrading enzymes (CAZymes) are arranged in discrete, physically linked and co-regulated gene clusters (Polysaccharide Utilization Loci; PULs) built around a pair of genes that encodes a glycan binding protein and a transporter [2,3]. Biochemical characterizations of the bespoke enzymes encoded in PULs have revealed that each PUL breaks down a particular glycan structure [4-6]. We reasoned that, reciprocally, the enzyme composition of PULs could inform on the structure of the targeted glycans. We have thus analyzed the composition of the PULs encoded by 959 genomes listed in PULDB [7] to estimate how many enzyme combinations have been elaborated by Bacteroidetes to degrade the natural diversity of glycans that they encounter in their various habitats.

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Large-scale screening of activities in unexplored CAZy sub-families, and distant or non-classified CAZymes

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Keywords: high-throughput production, functional characterization, CAZyme discovery

Despite the development of post-genomics methods such as transcriptomics, proteomics and metabolomics, the assignment of function to protein sequences remains one of the main challenges in modern biology. Taking advantage of the CAZy database, which classifies CAZymes into families and subfamilies based on amino-acid similarities, we successfully applied a strategy relying on a rational bioinformatics selection of enzyme targets, gene synthesis, high-throughput recombinant protein production and screening assays on a wide diversity of carbohydrate substrates.

We recombinantly produced 564 proteins selected from subfamilies without any biochemically characterized representatives (155 targets), from distant relatives of characterized CAZymes (222 targets) and from non-classified proteins that show little similarity with known CAZymes (187 targets). Sufficient levels of solubility were obtained for 332 proteins (59%). Screening these proteins for activity on a wide collection of carbohydrate substrates led to the assignment of a function to 38 proteins belonging to 25 distinct uncharacterized subfamilies (first set), to 23 distant proteins in 14 families (second set) and to the discovery of 13 novel CAZyme families (based on the activity of 19 proteins in the third set), two of which were also discovered by others during the course of our work. Given that, on average, six new GH and PL families are described every year, the work presented here therefore allowed the identification of about twice the number of new CAZyme families reported worldwide per year. Overall, 79 of our 332 soluble targets displayed enzymatic activity (24%). While all activities in subfamilies were already known in the family, seven of the distant targets were active on a substrate that had never been reported in the corresponding family. For example, an endo-β(1,4)-glucanase and a β-D-xylosidase activity are reported for the first time in family GH2. Moreover, we identified three previously unknown substrate specificities, that have never been described in any CAZy families before, among the newly discovered CAZyme families. Although the aim of this work was not to perform a detailed biochemical characterization of the enzymes, chromatographic and NMR methods were applied to characterize the end-products for some of the most original activities, including the first gellan lyase and an enzyme able to cleave the polysaccharide secreted by the cyanobacterium *Nostoc commune*. Interestingly, two new families are also found in Fungi, and deeper knowledge will likely arise for most of these new families since all, but one, appear in the intensively-studied Polysaccharide Utilisation Loci of Bacteroidetes.

With the decreasing cost of recombinant protein production, a similar approach conducted on thousands of targets would not only generate more discoveries, but would also enable a more reliable, knowledge-based functional prediction for gene products from genomic or metagenomic sequencing projects, with the key to success being the availability of a large library of substrates.

Ultra-high-throughput discovery of dietary and host glycan utilization pathways in gut microbiomes

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Keywords: microbiome, microfluidics, human glycans, glycoside transporters.

Glycans are produced by all living cells and are keystones in metabolism. They also play a fundamental role in the interactions between eukaryotes, bacteria and even viruses. Microorganisms face glycan structural complexity and diversity by producing a broad range of glycan utilization machineries, involving carbohydrate transporters, synthesizing and degrading enzymes. In order to accelerate the discovery of these glycan-metabolizing pathways from mammal gut microbiomes, we have developed versatile high- and ultra- high throughput functional metagenomics approaches, exploiting the last advances in nanotechnologies and microfluidics. These pipelines are compatible with any kind of gene libraries and allow us to screen Gbp of sequences in few hours with less than 1 mg of fluorogenic or native subtrates. We exploited them to explore the vast world of still uncultured microorganisms from various ecosystems, in particular the human gut microbiota, in order to decipher, up to the molecular level, the glycan-mediated host-microbiota-food interactions. We identified a panel of dietary, mucin glycan and ganglioside metabolizing pathways from commensal gut bacteria, including beneficial and non-beneficial species from different genera. The discovered loci, which encode a battery of sialidases, β-D-N-acetyl-glucosaminidases, β-D-N-acetyl-galactosaminidases, β-D-mannosidases, and also glycoside-phosphorylases and transporters from various families, are, for most of them, highly prevalent and abundant in the gut microbiome, and explain the metabolic flexibility of bacteria feeding both on dietary and human glycans. In addition to opening up new perspectives for the control of the microbiota functioning, the new enzymes and transporters identified represent biotechnological tools of interest for synthetic biology.

Discovery of new enzymes for universal donor blood production

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Keywords: deacetylase, galactosaminidase, metagenomics, universal donor blood

Blood transfusion is an indispensable part of the health care system, saving many thousands of lives annually. Although significant improvements in the collection and use of blood have been made over the years, there are always shortages in the supply of blood. A solution to this problem could lie in the enzymatic conversion of A, B or AB blood groups into universal donor red blood cells [1], suitable for all blood types. However, no economically reasonable enzymes that catalyze this process are currently available. Access to efficient enzymes that can convert A- and B-type red blood cells to universal donor O-type is urgently needed.

The human gut mucus layer harbors mucins, glycoproteins presenting O-glycan structures like the A and B blood antigens, which are foraged by gut microorganisms, an ideal source of unexplored carbohydrate-active enzymes. A functional metagenomic screening of this environment identified a novel enzyme pair from the obligate anaerobe *Flavonifractor plautii* that work in concert to efficiently convert the A-antigen to the H-antigen of O-type blood, via a galactosamine intermediate (Fig.1).

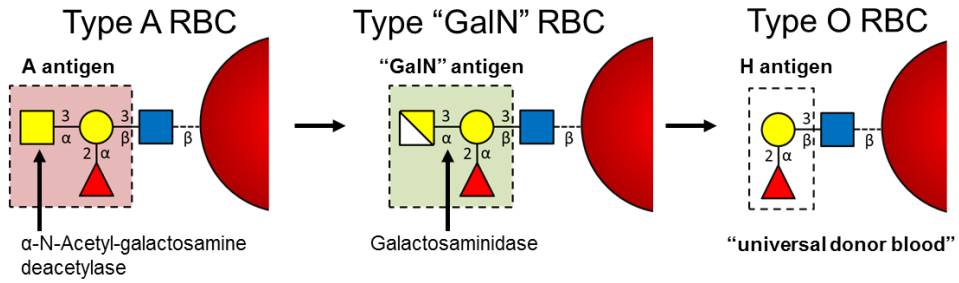

Figure 1. Overview of A two-step conversion from A red blood cells (RBCs) to O RBCs. A deacetylase converts the A antigen (GalNAc-α1,3-(Fucose-α1,2-)Gal-) to a galactosamine antigen (GalN-α1,3-(Fucose-α1,2-)Gal-) which is a substrate for the galactosminidase, creating the H antigen (Fucose-α1,2-Gal-).

The X-ray structure of the α-N-acetylgalactosamine deacetylase reveals the active site and mechanism of the founding member of this new CAZy family and the galactosaminidase represents a new activity within the CAZy family GH36. Their ability to completely convert A to O at very low enzyme concentrations in whole blood will simplify their incorporation into blood transfusion practice, broadening supply.

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Peptidoglycan deacetylases. Dual *N*-acetylglucosamine and *N*-acetyl-muramic acid specificities, structures and biological functions

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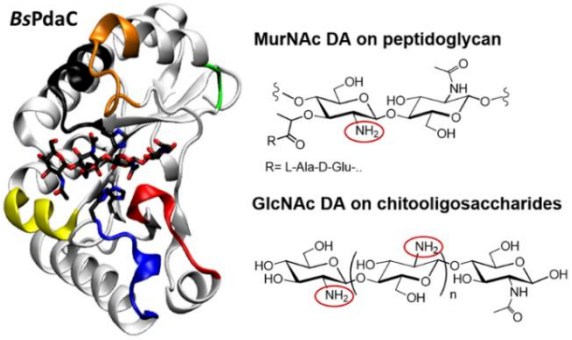
Keywords: peptidoglycan, CE4 enzymes, deacetylation, 3D structures

Deacetylation of their own cell wall polysaccharides is a strategy used by pathogenic bacteria and fungi to evade the host immune responses at initial stages of infection. Pathogenic bacteria utilize acetylation (6-*O*-acetylation of MurNAc) and deacetylation (2-*N*-deacetylation of GlcNAc and/or MurNAc residues) of their cell wall peptidoglycan (PG) to evade detection by the innate immune system. Likewise, plant pathogenic fungi partially deacetylate their cell wall chitin to be resistant to degradation by plant chitinases or deacetylate the released chitooligosaccahides (COS) to escape recognition by chitin receptors and evade the plant immune responses.

Peptidogycan and chitin deacetylases are members of family 4 carbohydrate esterases (CE4 enzymes) which operate by a metal-assisted general acid/base catalytic mechanism [1]. We are interested in understanding the structural bases of substrate specificity by CE4 enzymes, their mechanism of action and biological functions, as well as the use of engineered variants as biocatalysts. A number of PG GlcNAc deacetylases have been characterized and their 3D structures determined by X-ray crystallography. They are specific for GlcNAc residues in the PG chain but they have also shown to deacetylate COS. In contrast, few PG MurNAc deaceylases have been biochemically characterized. The *B. subtilis* PdaA deacetylates MurNAc residues of PG devoid of the peptide linked to MurNAc residues, which is consistent with its function during sporulation to form muramic acid δ-lactam residues in the spore cortex peptidoglycan. It is specific for MurNAc residues and it is not active on COS. Recently, a novel peptidoglycan MurNAc deacetylase, *Bs*PdaC, has recently identified [2], which deacetylates MurNAc residues of PG and, strikingly, it also deacetylates COS, an activity that was thought to be restricted to GlcNAc deacetylases.

To understand the molecular bases of such dual activity, we here report the biochemical characterization of *Bs*PdaC, its mode of action on chitooligosaccharide substrates, the X-ray 3D structure of the CE4 catalytic domain, and structural comparison with canonical MurNAc and GlcNAc deacetylases [3]. We propose that PdaC is the first member of a new subclass of peptidoglycan MurNAc deacetylases, with yet unknown biological functions, based on these differential functional and structural characteristics. Additionally, and based on the novel specificity on COS, these PG deacetylases are potential biocatalysts in combination with chitin deacetylases for the synthesis of partially acetylated COS with defined patterns of acetylation, current targets for a number of biotech applications [4,5].



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Structural and functional insights into the mode of action of a modular lytic polysaccharide monooxygenase

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Keywords: LPMO, dynamics, NMR, MD

Lytic polysaccharide monooxygenases (LPMOs) are copper-dependent enzymes that bind to the crystalline surface of polysaccharides (e.g. chitin and cellulose) and cause cleavage of β-1,4 glycosidic bonds by an oxidative mechanism. *Sc*LPMO10C is a cellulose-active LPMO that produces C1-oxidized chain ends (i.e. aldonic acids) [1]. This LPMO is composed of an N-terminal AA10 catalytic domain that is connected by a linker of approximately 30-amino acids with low sequence complexity to a C-terminal family 2 carbohydrate-binding module (CBM). Our current understanding of LPMO activity is much focused on the catalytic mechanism, while the role of CBMs and linker regions in LPMOs is still poorly understood. Here, we have used NMR spectroscopy [2] and SAXS, in combination with MD simulations, to probe the distinct dynamic characteristics of full-length *Sc*LPMO10C, revealing conformational flexibility of the linker as well as the overall structure and shape of the full-size protein. To the best of our knowledge, this is the first structure of a full-length carbohydrate-active enzyme with this type of extended flexible linker. We discuss how the structure and dynamic features of full-length *Sc*LPMO10C guide its biochemical activity.

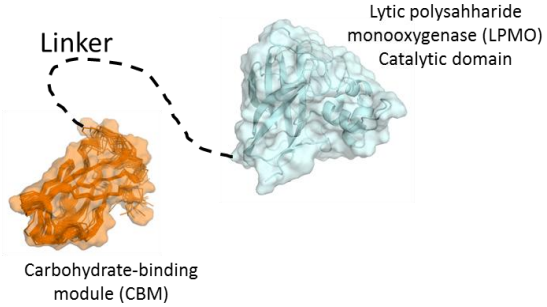

Figure 1. Model of full -length *Sc*LPMO10C showing the CBM2 carbohydrate-binding module (PDB ID: 6F7E) in orange, the AA10 catalytic domain (PDB ID: 4OY7) in cyan and the linker region as a dashed line.

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The human GDP-mannose-4,6-dehydratase reveals the minimal active site needed for NDP-sugar dehydration

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Keywords: β-elimination, reaction mechanism, enzyme catalysis, short chain dehydratases.

Biosynthesis of 6-deoxy sugars, including L-fucose, involves a mechanistically complex, enzymatic 4,6-dehydration of hexose nucleotide precursors as the first committed step. The basic enzymatic mechanism is highly conserved and involves three catalytic steps. Initially NDP-hexoses are oxidized at C4'' by a $NADP^+$ cofactor tightly bound to the enzyme. Water is eliminated from NDP-4''-keto-hexoses at C5'' and C6'' to form a NDP-4''-keto-hexo-5'',6''-ene intermediate. Reduction of this intermediate by NADPH at C6'' gives the product and regenerates $NADP^+$. Although the basic reaction course is understood mechanistic details of the β-elimination and the final reduction remained elusive. The human GDP-mannose 4,6-dehydratase was studied as a model protein. Determination of pre- and post-catalytic crystal structures at atomic resolution, in combination with molecular dynamics simulation, biochemical characterization, and in-situ NMR-experiments of wild-type and mutant enzymes reveal that concerted acid-base catalysis from only two active-site groups, Tyr179 and Glu157, promotes *syn* 1,4-elimination from an enol (not an enolate) intermediate. We also show that the overall multistep catalytic reaction involves least position changes of enzyme and substrate groups; and that it proceeds under conserved exploitation of the basic (minimal) catalytic machinery of short-chain dehydrogenase/reductases.

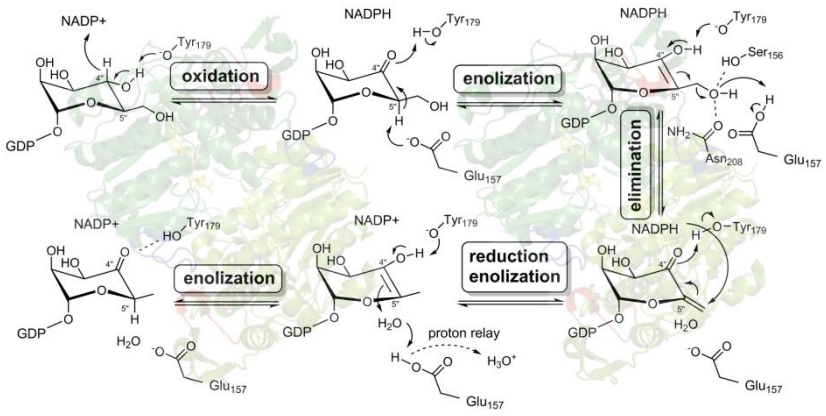

Figure 1. Proposal for the reaction mechanism of human GDP-mannose 4,6-dehydratase

Enzymatic cleavage of lignin-carbohydrate complexes by fungal glucuronoyl esterases

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Keywords: glucuronoyl esterases, lignin-carbohydrate complexes, CE15, aldouronic acids.

Glucuronoyl esterases (CE15 family) enable targeted cleavage of ester linkages in lignin-carbohydrate complexes (LCCs), particularly those linking lignin and glucuronoyl residues in xylan. We have shown for the first time a detailed product profile of an array of aldouronic acids released from a genuine LCC substrate from birch by a glucuronoyl esterase originating from the white-rot fungus *Cerrena unicolor* (*Cu*GE). The combined action of *Cu*GE and endo-xylanase GH10 significantly increased the product release compared to the action of the endo-xylanase alone. The data verify the enzyme's unique ability to catalyze removal of all glucuronoxylan associated with lignin via ester linkages, a function we suggest as crucially important for the fungal organism's ability to effectively utilize all available carbohydrates [1].

In addition, we have developed an assay using the insoluble LCC rich lignin fraction to determine the kinetic parameters of four fungal CE15 enzymes, *Ps*GE, *Cu*GE, *Tt*GE and *Afu*GE originating from lignocellulose degrading fungi *Punctularia strigosozonata, Cerrena unicolor, Thielavia terrestris* and *Armillaria fuscipes* which were identified by peptide pattern recognition amongst approx. 1000 putative CE15 protein sequences [2]. All four enzymes had activity towards the LCC containing lignin fraction and showed a clear preference for the insoluble substrate compared to smaller soluble LCC mimicking esters [3].

The four enzymes were also tested on a LCC rich substrate from spruce and reaction products were detected from all four CE15s [3]. This indicates that ester-linked LCCs are present i other types of biomass than hardwood and that GE activity is a generic type of activity used to degrade lignocellulose. In bioprocess perspectives, these enzymes would be clear candidates for polishing residual, contaminating carbohydrates from lignin to achieve a pure, native lignin fraction after minimal pretreatment.

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Engineering of the production of health-promoting glucosinolates in heterologous hosts

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Keywords: plant specialized metabolites, glucosinolates, pathway engineering, microbial production.

Glucosinolates are sulfur-rich specialised metabolites characteristic of the Brassicales order and well-known for their health-promoting effects upon intake of e.g. broccoli and cabbage. High amounts of broccoli and cabbage must be eaten on a regular basis to fully realize the health-beneficial effects and the current supplement market is based on products with unreliable amounts, if any at all. This has primed a strong desire to develop an efficient microbial cell factory for glucosinolate production as stable, rich source. We have engineered the production of 8-gene pathway of benzylglucosinolate into both *Escherichia coli* and *Saccharomyces cerevisiae*. In *E. coli*, we have systematically optimised the production levels by multiple ways which resulted in production from undetectable to approximately 4.1 µM benzylglucosinolate. Additional optimisation of pathway flux increased the production additionally 5-fold to 20.3 µM (equivalent to 8.3 mg/L) benzylglucosinolate. However, towards our goal of engineering microbial high level production of glucosinolates, we have encountered challenges related to the sulfur chemistry demand of glucosinolates and related to that pathway intermediates escape from the host cells resulting in prematurely abortion of the pathway. We apply pathway engineering as well as transport engineering approaches to overcome these challenges. Learnt lessons will be discussed.

Chemoenzymatic Synthesis of Thio-linked Heparinoid Polysaccharides for Anti-Cancer Applications

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Keywords: heparin; glycosidic linkage; heparanase inhibitor; synthase

Heparan sulfate (HS) and heparin have either proven or potential therapeutic utility in areas ranging from angiogenesis, inflammation, hemostasis, and cancer. Glycan bioactivity is conferred by intrinsic structural features including disaccharide composition and sulfation pattern. In the last decade, the *in vitro* enzymatic synthesis of defined sugar chains with glycosyltransferase, epimerase, and sulfotransferase enzymes has made great strides towards generating patterns with various bioactivities. This selective synthetic control is required as the potent anticoagulant heparin cannot be employed for some promising treatments due to the concerns for excessive bleeding at the needed dose; thus new structures without the key sulfates for binding with antithrombin or Factor X are needed.

Malignant cells employ a heparanase to cut HS and thereby steal growth factors from normal tissue and create highly angiogenic HS species to increase the tumor's blood supply. The chemical blockade or genetic knock-out of heparanase has been shown to reduce disease by a variety of cancer types. Heparin, while a potent heparanase inhibitor, cannot be used to treat cancer in humans in its original state due to its hemorrhagic risks.

In another synthetic step forward, we have re-engineered the glycosidic linkages between the HS sugar units with a goal of blocking heparanase processing. We show that an UDP-GlcNAc derivative with C4-thio substitution can be used as a donor by a HEP synthase from the microbe *Pasteurella multocida*, PmHS2, to add onto heparosan (HEP) chains [-4-GlcAβ1-4-GlcNAcα1-]. If the second required donor, UDP-GlcA, is also added to the reaction, then PmHS2 co-polymerized a novel HEP-like polymer with alternating '*S*-links' between sugar units rather than the entirely *O*-links of natural chains. Chains with dozens of *S*-links, [-4-GlcAβ1-***S***-4-GlcNAcα1-]$_n$, have been produced, the first example of such a polysaccharide reported thus far.

In analogy to the non-hydrolysable lactose promoter inducer, IPTG, we expect the *S*-links between sugar units will be resistant to enzyme-mediated cleavage thus this novel polymer backbone should be recalcitrant to the action of mammalian heparanase, a target for cancer treatment. We are on the road to create a non-anticoagulant, stable *S*-linked HS analog that should serve as a competitive inhibitor for heparanase. In comparison to existing heparanase-inhibitor candidates, our material is not animal-derived and possesses a more natural, non-immunogenic structure.

Glycosidase-catalyzed synthesis of glycosyl esters and phenolic glycosides

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Keywords: enzymatic glycosylation, rutinosidase, carboxylic glycoside, coumaric acid

Glycosides of phenolic acids and of derivatives of hydroxycinnamic acids are ubiquitous in plants, however their isolation from plant material is very tedious. Most of phenolic acid glycosides are glycosylated on the aromatic hydroxyls. However, glycosides attached to the carboxylic moiety can also be rarely found (glycosyl esters; typically β-glucopyranosides). Their chemical synthesis is not trivial and involves inherent problems of high lability of glycosyl ester bond, which is incompatible with most acyl protection groups.

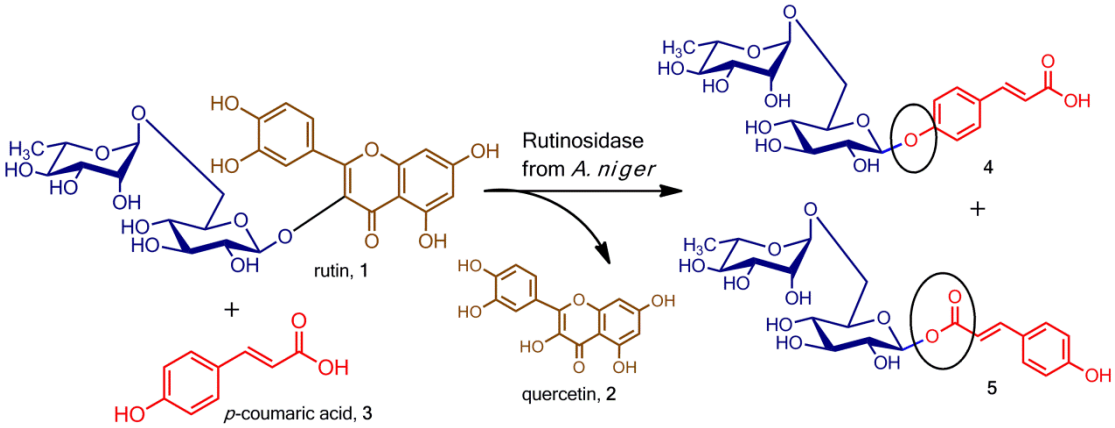

Figure 1. Rutinosylation of *p*-coumaric acid (**3**) with rutinosidase from *A. niger*.

Enzymatic approach mimicking *in vivo* biosynthesis employs glucosyltransferases but this method uses expensive UDP-glucose and the yields are low. We have recently isolated new robust diglycosidase rutinosidase from *A. niger*, which is able to glycosylate various acceptors including phenols [1] in a good yield using cheap rutin (**1**) as a glycosyl donor. To our great surprise glycosyl esters were also formed at a reasonable yield. We tested this reaction with a large panel of various phenolic acids and as an example we demonstrate rutinosylation of *p*-coumaric acid yielding phenolic glycoside (**4**) and respective glycosyl ester (**5**). A broader application of this new type of reaction was demonstrated by the synthesis of respective glycosyl esters of *p*-, *m*-, *o*-coumaric acids, ferulic acid and others. Rutinosides can be treated *in situ* with α-L-rhamnosidase (*A. terreus*) to yield respective β-glucopyranosides. We describe here probably the first example of glycosylation of a carboxyl group with a glycosidase.

Acknowledgement: Czech Science Foundation project 18-00150S and the joint Czech-Italian AVČR-CNR (V.K. & S.R.) mobility project No. CNR-16-30 are acknowledged.

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Metabolic and enzyme engineering to produce high quantities of the steviol glycoside Rebaudioside M in *S. cerevisiae*

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Keywords: Rebaudioside M, UDP-glycosyltransferase, *S. cerevisiae*, stevia

Amyris is an integrated renewable products company that produces sustainable ingredients for the health & wellness, clean beauty, and flavors & fragrances markets. Amyris applies its innovative bioscience platforms to convert plant sugars into a wide variety of molecules. One molecule in Amryis's portfolio is the zero-calorie sweetener Rebaudioside M (Reb M, Figure 1.) RebM is found naturally at low concentrations in the plant *Stevia rebaudiana*, from which the artificial sweetener stevia is derived. RebM is at least 300 times sweeter than sucrose and does not have the metalic or licorice flavors typically associated with stevia extracts and preparations. In fact, the taste profile of RebM is so remarkably similar to sucrose that RebM is the most desirable zero-calorie sweetener on the market today. However, RebM comprises at most 2% of the total steviol glycosides produced by the stevia plant, making purification difficult and expensive. We have taken an alternative approach, engineering a yeast strain to convert sugar cane syrup to pure RebM. In December of 2018, Amyris launched RebM as our 7th commercial ingredient, for use in food applications.

This talk will focus on the pathway optimization and enzymatic challenges the team overcame to produce pure RebM at high titers. The talk will discuss identification of a novel plant UDP-glycosyltranferase, a key breakthrough to producing high-purity RebM, as well other UDP-glycosyltransferases that were evolved for higher activity.

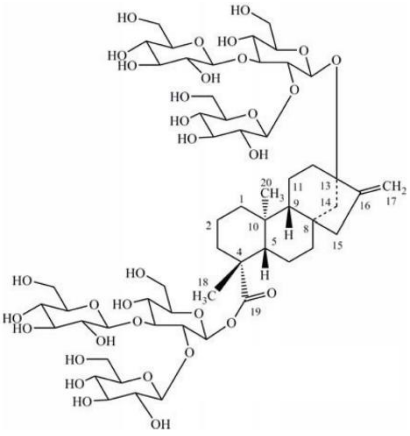

Figure 1. Rebaudioside M contains a 20-carbon isoprenoid core (called steviol). The carbohydrate structure is Glcβ(1-2)[Glcβ(1-3)]Glcβ1-O attached to the hydroxyl at the 13 carbon and Glcβ(1-2)[Glcβ(1-3)]Glcβ1-O attached to the carboxylic acid at the 19 carbon.

Tools for structural bioinformatics; design of a chimeric GH10 CBM-containing hydrolase

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Keywords: Comparative Modeling, Structure-based design, Chimeric enzyme, Glycoside Hydrolase.

Computer-assisted design is a valid route to inform protein engineering with a view to modify and/or derive novel functions. This talk is structure in two parts. In the first part of the talk I will be presenting two different tools developed in the lab to address different aspects of structure-based protein modeling and design. *M4T*, a method developed to predict the 3D structure of proteins and protein complexes based on multiple templates[1] and *Frag'R'Us*, a method devised for de novo fragment design[2]. In the second part of my talk I will presenting our results on the structural design and validation of a chimeric, multifunctional, enzyme. The starting point of this project was a novel GH10 hydrolase containing two CBM domains (Figure 1). The 3D structure of the enzyme was derived using comparative modeling allowing us to identify and define the boundaries of the different protein domains. Upon defining these, the CBM domains were substituted by two different functional domains: a GH43 hydrolase and a CE1 esterase. Several designs were derived and assessed by computational means to subsequently been cloned, expressed and tested *in vitro*. Experimental data showed that chimeric enzymes were active and bifunctional.

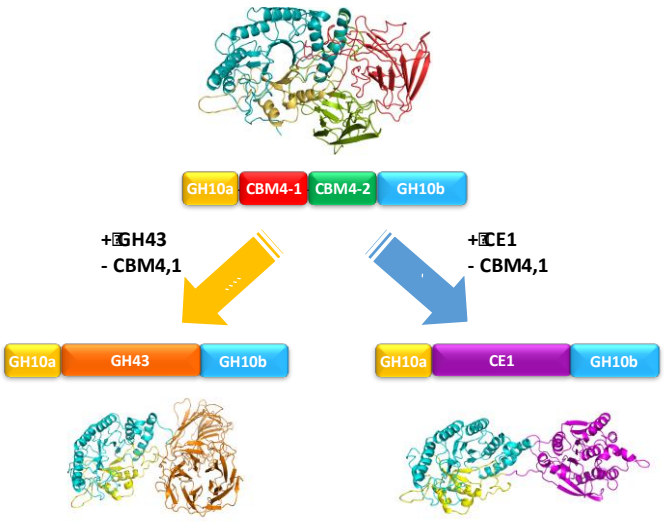

Figure 1. Design strategy of chimeric GH10 hydrolase. The removal of CBM domains on the native GH10 (top) were substituted by two different functional domains: GH43 and CE1, generating two novel chimeric enzymes (bottom). Structural models are shown in ribbon representations. Domain colored-coded as shown in the box representation.

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Efficient conversion of GHs into transglycosylases: a conservation-based approach

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Keywords: glycosylation, sequence conservation, mutations, oligosaccharide synthesis

Progresses in glycosciences are hampered by the lack of defined carbohydrates. This stems from the structural complexity of carbohydrates: they can be branched, in pyranose or furanose forms, connected through α- or β-configurations, and present diverse regioselectivities. In organic chemistry, it translates to multiple synthetic steps, resulting in poor yields and high amounts of waste. Protein engineering of Glycoside Hydrolases (GHs), to transform them into "green" glycosylation catalysts is a very promising approach. Its drawback lies in the time invested to develop a catalyst for each single reaction. However, once a suitable enzyme variant has been brought about, it allows for an environmentally friendly, low cost synthesis of the target. Enzymatic understanding and new approaches to efficiently generate desired biocatalysts are thus crucial to the development of glycosciences.

Here we propose and applied a conservation-based strategy, consisting in mutating conserved residues in the vicinity of the −1 subsite of GHs into their closest analogs (e.g., Tyr into Phe). Catalytic residues (when known), glycines and prolines are excluded. It should be noted that three-dimensional structures or biochemical information (such as mechanism or identified catalytic residues) are not needed. The advantages of this approach are two-fold: it is fast, as only about ten variants have to be assessed; and secondly, since the targeted residues are conserved, it allows for mutation transfer. Indeed, in GH families presenting different regioselectivities or acceptors specificities, transglycosylation yield improvement is likely transferrable, as acceptors subsites are not modified.

Over half of our produced enzyme variants from GH families 1, 2, 20, 29, 36, and 51 showed significantly enhanced transglycosylation yields (2 to 10-fold compared to native enzymes). Consequently, the approach has proven successful for the transfer of Gal, Glc, GlcNAc, GalNAc, Fuc, Man, or Ara monomers, D/L configurations, pyranose/furanose forms, and α/β stereochemistry. We obtained yields in the 50 - 80% range for the synthesis of such oligosaccharides as Fuc(α1,3)GlcNAc, Lacto-*N*-biose, Lacto-*N*-triose II, Arabinoxylooligosaccharides, and various other disaccharides.

Acknowledgments: Novo Nordisk Foundation supported this work.

BindScan: a computer-assisted protein design algorithm with applications in re-engineering CAZymes.

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Keywords: enzyme design, computational, glycosidases, transglycosylation.

In the perspective of using enzymes as biocatalysts to produce non-natural compounds, the starting substrates are usually non-natural as well. Since the enzyme-substrate interactions are not naturally-optimized in such cases, it is expected to be much room for the improvement of the catalytic efficiency. Identifying the regions of the enzyme structure most sensible to the binding of a given non-natural substrate is crucial for the redesign of the enzyme.

We will present here a computational algorithm (BindScan) that exhaustively casts all the positions on a given protein structure by individually mutating each position and measuring the effect on the binding affinity to a given compound. The positions of those mutants showing an improvement of the binding affinity with respect to the wild-type enzyme are considered as "hot-spots" sensible to the binding of the new compound. This information can then be used to experimentally design single point mutations or to guide directed evolution experiments for the improvement of substrate specificity in the working enzyme.

Different benchmarks of the algorithm on CAZymes will be discussed based on mutational data already published for GH13 and GH16 glycosidases. An application [1] of BindScan to the rational engineering of a GH51 arabinofuranosidase in to an efficient transglycosylating enzyme will be presented.

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Enzymatic glucosylation of a chemically-protected tetrasaccharide to access antigenic oligosaccharides

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Keywords: enzymatic glucosylation, carbohydrate-active enzyme, enzyme engineering, glycovaccine

In *Shigella flexneri*, a family of Gram negative bacteria responsible for bacillary dysentery, surface polysaccharides and glycans were identified as major targets of protection against reinfection. Access to these complex carbohydrates has been hampered by the lack of efficient synthetic tools. Here, we propose a synergistic strategy combining the use of chemical synthesis with biocatalysts such as carbohydrate-active enzymes as powerful tools to access glycobricks, easy-to-assemble into biologically active molecules mimicking the antigenic polysaccharide moiety – O-antigen (O-Ag) – of the *Shigella flexneri* lipopolysaccharides.

To access these glycobricks, regio- and stereo-specific glucosylation of a chemically-protected tetrasaccharide scaffold constitutes the first major step. However enzymes from the natural diversity do not necessarily display the required properties for glucosylation of non-natural carbohydrates and often need to be engineered. Several advances in the field have been made and mono- and di-saccharides could be glucosylated [1-5].
In this work, we report the successful enzymatic regio- and stereo-specific glucosylation of this chemically-protected tetrasaccharide with the use of branching sucrases from the GH70 family. These enzymes use a cheap and readily-available glucosyl donor substrate, sucrose, to catalyze naturally the glucosylation of glucans and they have also been shown to be highly tolerant toward their acceptor substrate. Within the frame of this work, this promiscuity has been further extended using computer-aided enzyme engineering techniques that enabled to produce a variety of glycobricks of interest to produce *Shigella flexneri* related oligogosaccharides.

This work was funded by the French National Research Agency (ANR Project CarbUniVax ANR-15-CE07).

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Completing the pathways of polysaccharide metabolism by marine heterotrophic bacteria

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Keywords: polysaccharide utilization loci, red algal galactan, glycoside hydrolases, metabolic pathways.

Macroalgae contribute substantially to primary production in coastal ecosystems. Their biomass, mainly consisting of polysaccharides, is cycled into the environment by marine heterotrophic bacteria (MHB), using largely uncharacterized mechanisms. Among the marine polysaccharides, carrageenans, alongside agars, are the main cell wall polysaccharides of red macroalgae and, besides being part of the environmental carbon source, play vital roles in the development and physiology of these photosynthetic eukaryotes. These complex polymers consists of D- and L-galactose based units alternatively linked by β-1,4 and α-1,3 linkages. The β- linked unit is either a D/L-galactose-6-sulfate or a 3,6-anhydro-D/L-galactose, a bicyclic sugar unique to red macroalgae [1]. The marine heterotrophic Bacteroidetes *Z. galactanivorans* is rich in CAZymes and we have explored and discovered missing links in the catabolic pathways of red algal galactans [2,3]. While in Bacteroidetes these degradation systems are encoded in PUL, other genera of marine bacteria also contain key features dedicated to polysaccharide catabolism. For example, we recently identified an entire plasmid dedicated to carrageenan degradation in the γ-proteobacterium *P. carrageenovora* [4]. By comparing enzymes and their modes of action, such as GH16 k-carrageenases for example, possible niche specific traits can be inferred, compatible with the respective life-styles of these bacteria. In a more general context, the results allow for an extension on the definition of bacterial PUL-mediated polysaccharide digestion [3].

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Prevotella beta-Mannan Utilization Loci: From the Rumen to the Human

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Keywords: Polysaccharide Utilization Locus, mannan, Prevotella, metabolism.

β-mannans are a diverse family of plant structural and storage glycans that comprise a significant part of human and livestock diets. However, aspects of β-mannan utilization in animals remain unclear, including the diversity of molecular systems devised by gut microbiota to address these complex carbohydrates. Polysaccharide Utilization Loci are complex, contiguous gene clusters deployed by diverse members of the Bacteroidetes phylum to address individual hetero-polysaccharides [1]. We present here the identification and detailed functional characterization of a large β-mannan utilization locus (β-MUL) from the historically important ruminant bacterium *Prevotella bryantii* B_14, using combined bioinformatic, biochemical, and structural biological approaches. We demonstrated that this locus encodes the complete repertoire of glycan-binding and catalytic activities necessary for the saccharification of (galacto)(gluco)mannans, which the host animal genomes otherwise lack. Holistic understanding of *Prevotella* β-MUL function and organization enabled the identification of diverse putative β-MULs across the Bacteroidetes phylum based on a defining mannobiose-specific symporter-epimerase-phosphorylase triad as a molecular marker. Moreover, we were able to use the ruminal *P. bryantii* B_14 β-MUL as a query to identify homologous sequence signatures among cattle (rumen) and human (monogastric) gut metagenomes, thereby indicating the concordant distribution of *Prevotella* across these diverse niches.

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The *Ruminococcus bromii* amylosome: Structure of the Amy12 pullulanase and its starch-binding protein partner Doc20

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Keywords: Starch, microbiota, amylosome, CBMs

Resistant starch (RS) describes the portion of starch that cannot be digested by host intestinal glucoamylases and therefore becomes food for human gut bacteria. RS changes the structure and metabolic output of the gut microbial community and tends to elicit a higher production of butyrate, a short chain fatty acid with anti-inflammatory and anti-tumorigenic properties. Therefore there is therapeutic potential in understanding the discrete ways gut bacteria target RS. While many gut bacteria encode glycoside hydrolase family 13 (GH13) enzymes in their genomes, very few gut bacteria can break down RS. *Ruminococcus bromii* has the unique ability to degrade granular RS via the expression of an amylosome, a multiprotein complex that somewhat parallels the cellulosome in assembly yet targets starch [1]. A goal of our research is to understand the protein players within the amylosome and how these proteins synergize to degrade RS in the gut environment. Amy12 is a pullulanase component of the amylosome. We have determined the x-ray structure of this enzyme in complex with maltoheptaose, 6^3-α-D-glucosyl-maltotriose and 6^3-α-D-glucosyl-maltotriosyl-maltotriose demonstrating the molecular basis of pullulan recognition and the flexibility within this active site to recognize α1,6-branching of starch. To determine what other proteins Amy12 may be interacting within during starch catabolism we performed co-immunoprecipitation of Amy12 using custom antibodies and found a single protein, termed Doc20, that co-purifies. Doc20, like Amy12, has a dockerin domain that presumably also binds to a cohesin within the amylosome complex. Beyond the C-terminal dockerin, Doc20 is comprised of two discrete domains that each bind starch. The crystal structure of the first domain of Doc20 displays two β-sandwiches, akin to two CBMs, arranged such that starch-binding is shared between these modules and can only recognize linear maltooligosaccharides but not cyclodextrins. Together these structures provide a first view of the enzymatic and starch-binding components of the *R. bromii* amylosome for RS digestion, and reveal new motifs in starch recognition in human gut bacteria.

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Novel insights into mucin degradation by key members of the human gut microbiota

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Keywords: mucin, O-glycans, microbiota, glycoside hydrolase family 16, *Bacteroides*, *Akkermansia*.

The human gut microbiota is closely associated with health, development and disease. Dietary and host glycans are major nutrient sources for these symbionts and different proportions of these can influence the composition of the microbiota. The thick mucus layer in the human colon is the key barrier between the contents of the lumen and the epithelial cells, providing protection against potential pathogens and environmental insults. A dense abiotic layer protects the cells, but a thinner upper layer is a niche for a subset of the microbiota. During a low fibre diet, the depletion of the mucin layer can be seen in parallel with an increase in the proportion those species that favour it as a nutrient source [1]. Despite the importance for gut health, the mechanisms used to breakdown mucin by the microbiota are not fully understood.

Genes encoding CAZymes were highlighted in upregulation data for several species of *Bacteroides* and also *Akkermansia muciniphila* [1,2]. Here we describe the glycoside hydrolase family 16 (GH16) enzymes with endo activity on polyLacNAc chains of mucin glycans. Sulfation and branching fucosylation could be accommodated in this activity for all enzymes and more complex decoration. Crystallographic data for several of these enzymes shed further light on their specificities. In addition, we are attempting to directly apply these enzymes to investigating human colon diseases, such as ulcerative colitis.

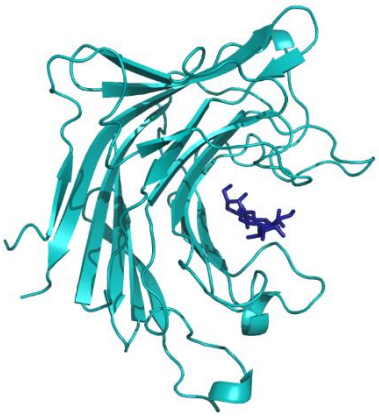

Figure 1. Crystal structure of a GH16 from a member of the human gut microbiota involved in the degradation of mucin.

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Structural analysis of β-L-arabinofuranosidases in GH127 and GH146

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Keywords: glycoside hydrolase, crystal structure, β-L-arabinofuranosidase.

Since GH127 was created according to the discovery of β-L-Ara*f*-ase HypBA1 from *Bifidobacterium longum* [1], ~2,500 ORFs are currently classified into GH127 and its related family, GH146. However, only a few enzymes in these families have been characterized. We have previously reported the crystal structure of HypBA1 complexed with β-L-Ara*f*, representing the product complex state [2]. The active site has a unique Zn^{2+} center coordinated by Cys×3 + Glu, and Cys417 was suggested to be the nucleophile residue. To further understand the structural features and the reaction mechanism of GH127-GH146 enzymes, we performed crystallographic analysis using various protein sources and soaking ligands.

GH146 is an interesting family that contains hundreds of bacterial ORFs and dozens of ORFs from plants and fungi whilst only one β-L-Ara*f*-ase (BT0349) has been characterized. We determined the crystal structures of two GH146 β-L-Ara*f*-ases, BLL3 (BLLJ_1848) from *B. longum* and XCV2724 from *Xanthomonas euvesicatoria* [3]. The Zn^{2+} binding motif was completely conserved in these enzymes while they have unique structural element covering the active site. To examine the hypothetical reaction mechanism of HypBA1, a synthetic substrate (*p*NP-β-L-Ara*f*) and site-specific probes for Cys (haloacetamide derivatives of L-Ara*f*) were soaked into HypBA1 crystals. We obtained a crystal structure of an acid/base catalyst residue mutant (E388Q) complexed with *p*NP-β-L-Ara*f*, representing the Michaelis complex state. A complex structure with BrAc-β-L-Ara*f* revealed that the amide-β-L-Ara*f* moiety was covalently attached to Cys417.

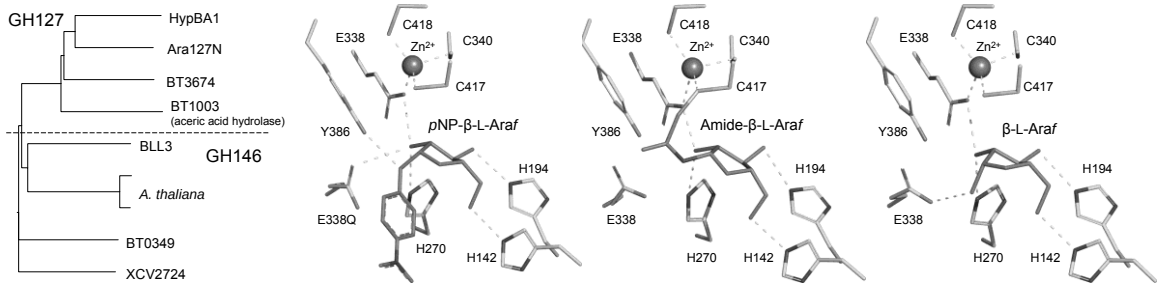

Figure 1. Phylogenetic tree of GH127-GH146 (left) and active site structures of HypBA1 (right).

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Early stages of glycogen biosynthesis: mechanism of action of glycogenin

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Keywords: glycogen biosynthesis, glycosiltransferases, S_Ni mechanism, metadynamics.

Biosynthesis of glycogen, the essential glucose (and hence energy) storage molecule in humans, animals and fungi, is initiated by glycosyltransferase enzyme glycogenin (GYG). The enzyme acts as a 'seed core' for the formation of the glycogen particle by catalyzing its own stepwise auto-glucosylation from a covalently-bound initiation site at Tyr195. To date, an inability to access homogeneous glycoforms of this protein, which unusually acts as both catalyst and substrate, has precluded precise mechanistic studies, particularly of the earliest catalytic intermediates. Recently, we have been able to recapitulate and understand mechanistic activity at different stages using a combination of palladium-mediated enzyme activation and molecular dynamics (MD) simulations [1], showing that there are different mechanistic phases, some of which can incorporate sugars different from glucose. We showed that the chemical reaction can be described as a "front-face" type, as previously found for other retaining glycosyltransferases [2,3], and that the donor and acceptor glucose units of GYG are optimally positioned for catalysis, resulting in a extremely fast glucosyl transfer.

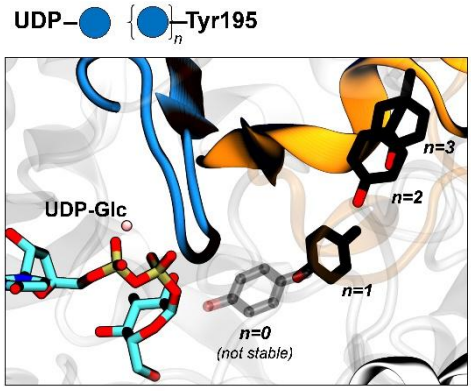

Figure 1. Motion of Tyr195 to accommodate acceptor substrates of various lengths. Results obtained from MD simulations of the corresponding GYG Michaelis complexes.

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Structural and Mechanistic Basis for Peptidoglycan O-Acetylation in both Gram-Positive and Negative Pathogens

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Keywords: Peptidoglycan, *O*-acetyltransferases, reaction kinetics, X-ray crystallography.

The O-acetylation of the essential bacterial cell wall polymer peptidoglycan (PG) occurs in many bacteria including important human pathogens, such as *Staphylococcus aureus*, species of *Enterococcus*, *Helicobacter pylori*, *Campylobacter jejuni*, and *Neisseria gonnorrhoeae* [1]. This modification to the C-6 hydroxyl of *N*-acetylmuramoyl residues in PG inhibits the action of the lysozymes of innate immune systems, and it totally precludes the activity of the lytic transglycosylases, bacterial autolysins required for the insertion of flagella, pili, and secretion/transport systems, as well as the general biosynthesis and turnover of the PG sacculus. We have characterized two distinct two-component systems for PG O-acetylation in Gram-positive and Gram-negative bacteria, respectively. In Gram-negative bacteria, such as *N. gonorrhoeae*, an integral membrane protein, PG *O*-acetyltransferase (Pat) A, is proposed to translocate acetate from cytoplasmic pools of acetyl-CoA through the cytoplasmic membrane to the periplasm for its transfer to PG by PatB [2]. With *S. aureus, S. pneumoniae* and other Gram-positive pathogens, a single protein, *O*-acetyltransferase (OatA), appears to be a fusion of PatA and PatB that catalyzes both the translocation and transfer of acetyl groups for PG O-acetylation [3]. Herein, we present kinetic and structural insights that explain why these enzymes would function strictly as *O*-acetyltransferases *in vivo* rather than as esterases.

Steady-state kinetic analyses of *N. gonorrhoeae* PatB and the C-terminal catalytic domains of OatA from *S. pneumoniae* (*Sp*$OatA_C$) and *S. aureus* (*Sa*$OatA_C$) indicated that each use a ping-pong, bi-bi catalytic pathway for acetyl transfer to acceptor sugars. Pre-steady state analysis of the esterase activity of *Sp*$OatA_C$ revealed that the rate-limiting step for the hydrolytic reaction was the breakdown of the acetyl-enzyme with a half-life of > 1 min. A novel PG-based substrate was used to delineate the unique specificities for the $OatA_C$ enzymes which account for their different temporal activities in PG metabolism. The X-ray crystal structures of all three enzymes adopt an α/β hydrolase fold comparable to SGNH esterases, and a Ser-His-Asp catalytic triad was identified within the active site grooves on the surface of each enzyme. However, unique oxyanion loop orientations compared to the SGNH esterases were found. Site-specific replacements confirmed the identification of these catalytic residues. The structure of *Sp*$OatA_C$ was also determined in complex with a mechanism-based inhibitor covalently bound to the catalytic Ser. A mechanism of action is proposed for these PG *O*-acetyltransferases involving the formation of an acetyl-enzyme intermediate prior to the acetylation of *N*-acetylmuramoyl residues in PG.

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Findings of new CAZymes, β-1,2-glucan-associated enzymes

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Keywords: β-1,2-glucan, 1,2-β-oligoglucan phosphorylase, β-1,2-glucanase, β-1,2-glucooligosaccharide.

Functions and structures of CAZymes have widely evolved in correspondence with wide variety of carbohydrate structures. Though new CAZymes and glycoside hydrolase families (GHs) have been found continuously, there still remains many CAZymes with unknown functions even in the case of enzymes acting on glucose polymers. β-1,2-Glucan is a polysaccharide found in nature (mainly known as a cyclic form produced by Rhizobia). However, no β-1,2-glucan-degrading enzyme has been identified until recently.

First, we found a new glycoside phosphorylase acting on β-1,2-glucan (1,2-β-oligoglucan phosphorylase, SOGP) in GH94 [1,2]. SOGP was used for a large-scale preparation of β-1,2-glucan [3]. A β-1,2-glucan-degrading bacterium was cultured using the synthesized β-1,2-glucan as a sole carbon source, leading to identification of a β-1,2-glucanase (SGL) and creation of a new GH (GH144) [4]. In GH144, a unique SGL releasing sophorose from β-1,2-glucan was also found [5]. A fungal SGL was first identified to be an eukaryotic enzyme that should be classified into a new GH. This enzyme has a unique reaction mechanism that both general acid and general base are non-canonical. A β-1,2-glucooligosaccharide-binding protein and β-1,2-glucosidases preferable for β-1,2-glucooligosaccharides were found in gene clusters of *sogp* gene and *sgl* gene [6-8]. We will indroduce findings on structures and functions of these β-1,2-glucan-associated enzymes and protein.

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Discovering and Characterizing Alginate Lyases from Human Gut Microbiota

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Keywords: alginate lyases, polysaccharide lyase families 6 and 17, *Bacteroides cellulosilyticus*, *Bacteroides eggerthii*

Alginates are a linear anionic polysaccharides composed of 1,4 linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) arranged in homo- or MG mixed blocks. The global annual production approaches 38,000 tons. Alginate is consumed by humans in the form of food hydrocolloids, edible seaweeds and encapsulation of probiotics, nutraceuticals and drugs. Humans lack alginate degrading enzymes; however certain *Bacteroides* can catabolise alginate in the gut to health beneficial short chain fatty acids [1]. To date no enzymes from human gut microbiota capable of degrading alginate have been described. In the present work human gut *Bacteroides* alginate lyases belonging to polysaccharide lyase (PL) families 6 and 17 (PL6, PL17) are identified and characterized. The two PL6 enzymes, *Bcel*PL6 and *Beg*PL6 cleave polyM and polyG blocks, respectively. *Bcel*PL6 is a monomeric endo-acting enzyme strictly specific for polyM, while *Beg*PL6 releases monosaccharides from polyG blocks, but shows an endo-action mode on alginate and polyM. *Beg*PL6 and *Bcel*PL6 are monomeric in contrast to the only published alginate lyase structure in PL6 from a marine bacterium and with polyG preference [2]. The crystal structure of *Bcel*PL6 was solved to a resolution of 1.3 Å. It adopts a parallel β-helix fold and has only a single domain. A calcium serves as the neutralizing charge and the catalytic K249 and R270 are located in the active site along with a conserved H271. *Bcel*PL6 H271N has no activity but can be rescued by addition of imidazole, indicative of a vital role of H271 in PL6 catalysis. *Beg*PL17 releases unsaturated monosaccharides from all three types of alginate block structures. Together *Beg*PL6 and *Beg*PL17 can completely degrade alginate into unsaturated monosaccharides and linearization of these into 4-deoxy-L-erythro-5-hexoseulose uronate is catalyzed by the novel enzyme *Beg*KdgF, a process that also occurs spontaneously. The results provide insight into biochemical functionality of alginate lyases providing a useful stepping stone for identifying the enzymes responsible for alginate utilization in the gut. This work is funded by the DCSR (Grant no. 1308-00011B).

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Fungal biodiversity: a tremendous resource for CAZymes discovery

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Keywords: filamentous fungi, biodiversity, lignocellulosic biomass, cellulose, CAZymes, LPMOs.

Filamentous fungi are among the most potent degraders of lignocellulosic biomass due to their ability to thrive in lignocellulose-rich environments; they produce a high number and a broad variety of carbohydrate-active enzymes (CAZymes) targeting the different components of biomass. Fungal biodiversity, collected from tree stumps in temperate and tropical regions, was explored using robotic methods to identify fungal strains that deconstruct efficiently recalcitrant polysaccharides. These investigations enabled the unbiased identification of fungal strains issued from biodiversity with high biotechnological potential.
To understand their mechanism of action, some of these fungal strains were further investigated using activity profiling and genomic, transcriptomic and proteomic techniques. The comparative analysis of fungal secretomes using proteomics highlighted the cooperation between fungal enzymes for enhanced degradation of complex lignocellulosic substrates and some discrepancies in CAZymes sets dedicated to different types of biomass.
Over the last ten years, hundreds of fungal secretomes gathering several thousands of lignocellulose-acting enzymes have been analyzed. Bioinformatic exploration of these post-genomic data coupled with functional data is a powerful asset for the discovery of new enzymatic functions. In this lecture, there will be a focus on the discovery of new CAZy families gathering fungal lytic polysaccharide monooxygenases (LPMOs) [1-4].

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POSTERS ABSTRACTS

Improved Profiling of Sialylated N-Linked Glycans by HPAE-PAD

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Keywords: biopharmaceuticals, protein glycosyliation, HPAE-PAD, sialylated glycancs

Profiling a glycoprotein's asparagine-linked (N-linked) glycans is an important quality control assay for determining the fidelity and consistency of recombinant therapeutic glycoprotein production. It is also an important assay when evaluating changes in production conditions, comparing expression systems, and comparing a biosimilar to an innovator's product. Good profiling requires high resolution separation of a glycoprotein's released glycans. High-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) is a well-established technique for this assay which is orthogonal to other techniques such as capillary electrophoresis and hydrophilic interaction liquid chromatography with fluorescence detection. HPAE-PAD is especially effective for separating sialylated glycans, but there is opportunity to improve resolution. We evaluated changes to commonly used HPAE-PAD conditions to improve resolution. HPAE-PAD separations of N-linked glycans typically use 100 mM sodium hydroxide with a gradient of sodium acetate at 30 oC. Using the N-linked glycans released from bovine fetuin, bovine thyroglobulin, bovine fibrinogen, and human alpha 1 acid glycoprotein, we evaluated the effects of temperature and sodium hydroxide concentration on resolution. We show that lowering temperature and increasing sodium hydroxide concentration improve resolution. An example of improved resolution potential new peaks identified under higher hydroxide concentration and lower temperature is shown in Figure 1. We also evaluated column format changes to reduce analysis time as well as sample and reagent consumption. For example, using a short column we are able to quickly evaluate the charge status of a glycoprotein's N-linked glycans. This may be an effective way to quickly screen the impact of changes in cell culture conditions on sialylation.

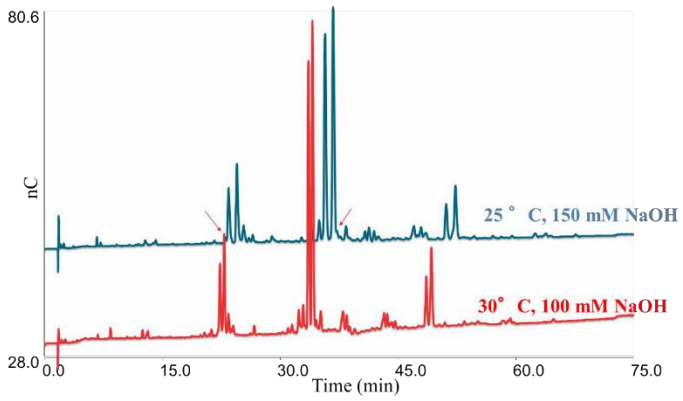

Figure 1. Fetuin alditols separated at high NaOH-low temperature condition compared to typical conditions (Note- potential new peaks identified under high NaOH-Low temperature condition are indicated by red arrows)

Influence of the carbohydrate binding module on the activity of an AA9 lytic polysaccharide monooxygenase

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Keywords: filamentous fungi, cellulosic substrates, LPMO, carbohydrate binding module.

Cellulose-active lytic polysaccharide monooxygenases (LPMOs) secreted by filamentous fungi play an important role in the degradation of recalcitrant lignocellulosic biomass [1]. They can occur as multidomain proteins fused to a carbohydrate-binding module (CBM). On a biotechnological point of view, LPMOs are promising and innovative tools for the production of nanocelluloses and biofuels [2] but their direct action on cellulosic substrates is not fully understood.

In this study, we probed the action of the family 1 CBM (CBM1) appended to the LPMO9H from *Podospora anserina* (*Pa*LPMO9H) using model cellulosic substrates. As expected, the deletion of the CBM1 weakened the binding to nanofibrillated cellulose, amorphous and crystalline cellulose. Although the release of soluble sugars from cellulose was drastically reduced under standard conditions, the truncated LPMO retained some activity on soluble oligosaccharides. The cellulolytic action of the truncated LPMO was demonstrated using synergy experiments with a cellobiohydrolase (CBH). Indeed, the truncated LPMO was still able to improve the efficiency of the CBH on cellulose nanofibrils in the same range as the full length LPMO. Analysis of the insoluble fraction of cellulosic substrates evaluated by optical and atomic force microscopy confirmed that the CBM1 module was not strictly required to promote the disruption of the cellulose network. Based on these results, we reduced the amount of water in the reaction to increase the probability of enzyme-substrate interaction in a CBM-free context. Increasing the substrate concentration enhanced the performance of *Pa*LPMO9H without CBM in terms of products release. Interestingly, removing the CBM altered the regioselectivity of *Pa*LPMO9H with a significant release of C1-oxidized products.

The absence of the CBM1 does not preclude the activity of the LPMO on cellulose but its presence has an important role in driving the enzyme to the substrate and releasing more soluble sugars (both oxidized and non-oxidized) therefore facilitating the detection of LPMO activity at low substrate concentration. These results will help us to guide the selection of suitable LPMOs for the production of nanocelluloses and biofuels.

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Structure and Function of Exopolysaccharide Modifying Enzymes in *Pantoea stewartii* Biofilm

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Keywords: *Pantoea* stewartii, biofilm, exopolysaccharide, stewartan, fluorescence correlation microscopy

Bacterial microorganisms growing in biofilms represent a severe pathogenic threat. The extracellular biofilm matrix surrounding the cells provides structural and mechanical support and controls penetration of metabolites, bacteriophages and antibiotics. *Pantoea stewartii* is a plant pathogen which infects maize and produces a viscous biofilm, leading to wilting of plants. The most abundant component in *P. stewartii* biofilms is the exopolysaccharide stewartan, consisting of anionic repeating heptasaccharide units and chain lengths of about 1-4 MDa (Figure 1).

The genes for the biosynthesis of stewartan are organized on gene loci *wce I-III*, encoding typical enzymes of the wzx/wzy/wzzz heteropolysaccharide synthesis and assembly pathway, but also for a gene with unknown function, *wceF*. We have isolated the protein WceF and analyzed its structure. Its domain organization resembles that found in bacteriophage tailspike proteins including a central β-helical fold [1][2]. Interaction studies with stewartan preparations revealed that WceF is a stewartan specific glycosyl hydrolase. We analyzed stewartan with fluorescence correlation spectroscopy and molecular dynamics simulations and found a diffusion limited exopolysaccharide network in which we further characterized exopolysaccharide digestion by WceF and bacteriophage enzymes.

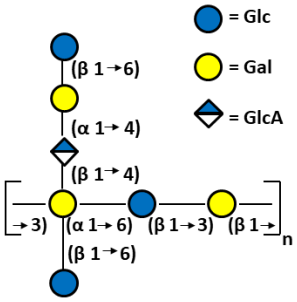

Figure 1. *P. stewartii* exopolysaccharide stewartan heptasaccharide repeating unit [3].

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Surface analysis tools identify how fungus *Aspergillus niger* modifies lignocellulose

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Keywords: lignocellulose matrix, surface analysis, fungal CAZymes, proteomics

Microbial carbohydrate active enzymes are exploited on an industrial scale as biocatalyst to convert plant lignocellulose to simple sugars. However, we have poor knowledge of the effect of microbes and their enzymes on the actual insoluble complex substrate, while such understanding underpins advancement of renewables-based biotechnology.

We aimed to understand how industrially relevant fungus *Aspergillus niger* and its enzymes interacts with lignocellulose, via investigating how exposure and accessibility of polysaccharides and lignin on complex lignocellulose surface changes during cultivation. Analysis of time-staged changes of the lignocellulose matrix, via a combination of mass-spectrometry based imaging (ToF-SIMS) and immunohistochemistry, identified increased surface exposure of lignin over time, and a differential degradation of hemicellulose and pectin polysaccharides. Degradation of specific polysaccharides was not always linked to presence of known corresponding degradative enzymes as identified by proteomics, suggesting lack of substrate access or absence of essential accessory enzymes.

Our results highlight that a full understanding of fungal and enzymatic lignocellulose degradation requires a combination of enzyme biochemical data with identification of modifications in real, complex lignocellulose matrices. This enables engineering of more effective biocatalysts and their exploitation in either break down of lignocellulose or modification to glyco-materials.

Mapping the Interaction site(s) of Alginate oligosaccharides on βeta-lactoglobulin

Beta-lactoglobulin (βLG) is a 18.3 kDa protein found in cow milk, that has extensively studied for its ability to bind hydrophobic ligands. It folds into nine β-strands along with an α-helix. The structure is contains to sulfur bridges but also has one free cysteine buried in the hydrophobic core. The structure of βLG is highly dependent on the pH of the environment. At low pH >3 βLG is mostly a monomer, at pH <3 it is shown to be a dimer and around pH 7.5 there is movement between the open and closed state of the calyx that can bind different hydrophobic ligands. Alginate oligosaccharides are short oligomers of alginate, alginate consist of a mixture of linked β-D-mannuronic and α-L-guluronic acid mixed and in block. These sugars are 1-4 linked and there is no system governing the composition of alginate. Alginate has a pKa around 3.2 but maintains a negative seta potential even at pH<pKa, this is one of the reasons why it is a Good binding partner to proteins. Both alginate and βLG is widely used as a food additive for gelling effects, pH modulation, water retention and antimicrobial activity, lately people have started becoming interested in the exact binding motif of the two in order to understand carbohydrate and protein interactions. Such a study was conducted with a trisaccharides derived from alginate, the study proved binding as well as location of binding sites. This study will go beyond that and work with tailored alginate oligosaccharides to investigate the difference between mannuronic acid, guluronic acid, the alternating structure as well as different lengths of polymers, from dp 4 to dp 6.

Lactococcus lactis Strain F-mou Exopolysaccharide: Production and Characterization

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Keywords: Exopolysaccharide, Mech-Degla Juice, Box-Behnken Design, *Lactococcus lactis*.

A new exopolysaccharide (EPS) was produced by the *Lactococcus lactis* strain F-mou (LT898177.1) isolated from the Sahrawi camel milk in the Bir-Naam region, Algeria. The most influential production parameters were screened by the Plackett-Burman design for enhancing EPS yield utilizing the Mech-Degla juice as a low-cost raw material. An optimum condition of a 0.49 of inoculum size, a 100 rpm of aeration rate, and a 12h of incubation period resulted in a 301 g/L. This yield was 47 times higher than the one attained before the application of the Box-Behnken Design. Additionally, the FTIR analysis of the EPS confirmed the presence of hydroxyl, carboxyl, amide and sulphate groups. Furthermore, the SEM image showed a porous structure characterized by a flake-like basic configuration with an extremely dense assembly. The NMR studies indicated that EPS contained a backbone of→4-α-D-galactopyranose-(1→, →4, 6-α-D-glucopyranose-(1→, →6- α -D- galactopyranose -(1→ linkages plus a levan part. The EPS exhibited good water and oil holding capacities, a high antioxidant efficiency, and an excellent anti-clotting activity. EPS also showed a strong inhibitory activity against *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Candida albicans, Bacillus cereus, Proteus mirabilis, Acinetobacter baumannii, Enterobacter cloacae*, and *Listeria monocytogenes*. Overall, the mentioned findings indicated that EPS could be utilized as a natural additive in pharmaceutical, food, and cosmetic industries.

Structure of solutions of carbohydrates and their derivatives, and their reactivity in glycosylation

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Keywords: supramers, light scattering, polarimetry, glycosylation.

We have been developing an approach based on hypothesis that reactivity of glycosyl donor and glycosyl acceptor, as well as stereoselectivity of glycosylation reactions in which they participate, are determined not only by molecular structures of reactants, but also by the structure of reaction solution [1]. Recently, the phenomenon of nano- [2] and mesoscale [3] heterogeneity of many macroscopically homogeneous solutions of low-molecular substances has been discovered and its importance for chemical reactivity emphasized [1]. We revealed the existence of "critical" concentrations that separate concentration ranges, in which solute can exist as various supramolecular objects (supramers [1], which can also include solvent molecules), differing in size and structure, hence physical and chemical properties [4-8]. Examples of how modulation of solution structure can influence the outcome of glycosylation will be presented. This work was financially supported by the Russian Science Foundation (Project No. 16-13-10244-P).

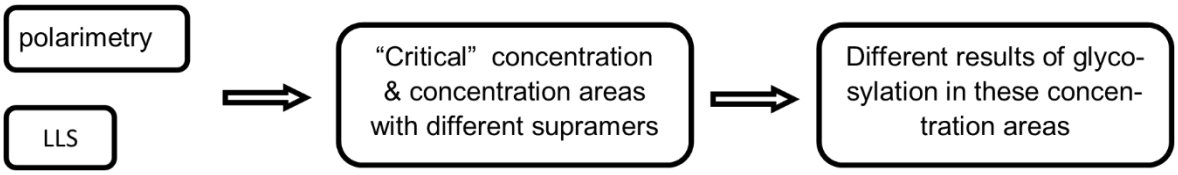

Figure 1. Supramer approach to glycosylation reactions.

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Investigation of the dynamics of lignocellulosic biomass enzymatic hydrolysis in 4D

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Keywords: lignocellulosic biomass, 4D imaging, enzymes, deconstruction, confocal microscopy.

Lignocellulosic biomass (LB) is a renewable resource from plants used as an alternative to fossil resources [1] and has a high potential to be converted into biomolecules, bioenergy and biomaterials without compromising global food security. However, the valorization of LB is still a challenge because of its complex structure and chemical composition making it recalcitrant to enzymatic deconstruction [2].

Different chemical, physical and spectral features have been investigated to understand LB recalcitrance and to predict its deconstruction [3-4-5]. However none of them seems to be universal but rather specific to biomass species and/or pretreatment. One key progress would be to assay some structural features at the cellular/tissular scale by evaluating the evolution of the 3D architecture of LB during enzymatic hydrolysis.

Therefore, we have devised an original confocal microscopy set-up to image the evolution of native and pretreated poplar samples during their hydrolysis. Samples have been reconstructed in 4D and different structural features have been quantified through newly developed segmentation and tracking algorithms. Results obtained show some markers are more relevant than some others to explain hydrolysis. Importantly, this sheds a new light on how enzymatic hydrolysis simultaneously impacts chemical and structural properties of LB samples.

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Xyloglucan degradation by Carbohydrate Active Enzymes from the hyperthermophilic archaeon *Saccharolobus solfataricus*

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Keywords: Glycoside Hydrolase, Hyperthermophiles, Hemicellulose

(Hyper)thermophiles are an excellent source for the discovery of new glycoside hydrolases (GH) playing a key role in the hydrolysis of the (hemi)cellulosic component. GHs, compared to others Cazymes, are a main target for enzyme discovery, particularly now that biorefining based on plant biomasses has become a major R&D pursuit [1].GHs isolated from (hyper)thermophilic organisms show many advantages over biocatalysts working at temperatures below to 50°C and demonstrated remarkable utility in the bioconversion of carbohydrates due to their high resistance to temperature, chemicals, and pH extremes [2].

In the hyperthermophilic archaeon *Saccharolobus solfataricus* several GHs have been identified and characterized and some of them are possibly involved in the degradation of Xyloglucan (XG) [3]. This hemicellulose comprises up to one-quarter of the total carbohydrate content of terrestrial plant cell walls and represents a significant reservoir in the global carbon cycle [4]. For its proprieties, XG and its chemically/enzymatically modified oligosaccharide derivatives has a wide range of applications [5].

Here we report on the characterization of hyperthermostable GHs from *S. solfataricus* possibly involved in the degradation of XG. These enzymes are active on XG oligosaccharides and are clustered in a region of 50 kb, suggesting that they could be involved in the hydrolysis of XG *in vivo*. The characterization of these enzymes on XG hydrolysis would allow to exploit their biotechnological potential.

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Alginate lyases from a polysaccharide utilization locus encoded by the gut bacterium *Bacteroides eggerthii*

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Keywords: Alginate lyases, *Bacteroides eggerthii*, polysaccharide utilization loci, polysaccharide lyase family 6 and 17

Alginate is an anionic linear polysaccharide consisting of the two uronic acids α-L-guluronic acid (G) and β-D-mannuronic acid (M). Within the polysaccharide chain M and G can be arranged in either blocks of M, blocks of G or mixed blocks. Alginate is present in edible seaweed and is a commonly used additive in the food industry. Humans do not encode enzymes for the degradation of alginate. However, certain *Bacteroides* strains present in the gut encode polysaccharide utilization loci (PULs) involved in the fermentation of alginate into health beneficial short chain fatty acids [1].

To date, the enzymes involved in alginate utilization in gut bacteria have not been studied in detail. In this study we present the two alginate lyases *Beg*PL6 and *Beg*PL17 from a polysaccharide utilization locus encoded by the gut bacterium *Bacteroides eggerthii*.

*Beg*PL6 is a monomeric enzyme, which is bispecific, degrading both polyM, polyG and polyMG blocks. *Beg*PL6 shows a preference for polyG and degrades these blocks into unsaturated monouronates whereas unsaturated oligosaccharides are produced when polyM or polyMG is used as the substrate. *Beg*PL17 releases unsaturated monouronates on all three block structures. Together, *Beg*PL6 and *Beg*PL17 can degrade alginate into unsaturated monouronates which can be further linearized into 4-deoxy-L-erythro-5-hexoseulose – a process which occurs either spontaneously or catalyzed by the novel enzyme *Beg*KdgF also encoded by the PUL. This work gives an insight into enzymatic degradation of alginate by gut bacteria and lays the foundation for further studies on alginate utilization in the gut.

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Carrageenan catabolism is encoded by a complex regulon in marine heterotrophic bacteria.

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Keywords: marine heterotrophic bacteria, polysaccharide utilization locus, red algae, carrageenan degradation

Macroalgae contribute substantially to primary production in coastal ecosystems. Their biomass, mainly consisting of polysaccharides, is cycled into the environment by marine heterotrophic bacteria using largely uncharacterized mechanisms. Carrageenans are a major component of the cell wall of red macroalgae. These sulfated polysaccharides have been widely used in various industries as gelling and texturizing agents. Carrageenans and their derived oligosaccharides have promising potential as bioactives (antivirals, anticoagulants, immunomodulators, etc.) presumably due to their resemblance to animal sulfated glycosaminoglycans. Ecologically, these polymers are a source of nutrients in coastal ecosystems, particularly for heterotrophic marine bacteria that are key players in the recycling of organic matter in the oceans. We have determined the complete catabolic pathway for carrageenans, in the marine heterotrophic bacterium *Zobellia galactanivorans*, using a combination of biochemical, crystallographic, transcriptomic, genetic and bioinformatics techniques [1]. Carrageenan catabolism relies on a multifaceted carrageenan-induced regulon, including a non-canonical polysaccharide utilization locus (PUL) and genes distal to the PUL, including a *susCD*-like pair. The carrageenan utilization system is well conserved in marine *Bacteroidetes* but modified in other phyla of marine heterotrophic bacteria, including the absence of the *susCD*-like pair. These results allow for an extension to the definition of bacterial PUL-mediated polysaccharide digestion and provide a major advancement towards understanding the biomolecular mechanisms governing the carbon cycle in coastal ecosystems.

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Expression of glycoside hydrolases from the brown-rot *Fomitopsis pinicola*

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Keywords: brown-rot, *Fomitopsis pinicola*, glycoside hydrolases, lignocellulose degradation

Renewable products derived from plant biomass are emerging as an important field of biotechnological applications. Nevertheless, the recalcitrant and heterogenic nature of lignocellulose causes the deconstruction and fractionation to be the principal bottlenecks for its utilization. In nature, enzymatic degradation is achieved by the secretion and collective action of multiple carbohydrate-active enzymes [1]. However, a profound understanding of the decomposition mechanisms and interactions is required for its industrial application. In this context, the ERC-COG OXIDISE focuses in the role of fungal extracellular enzymes from the brown-rot *Fomitopsis pinicola* as biocatalysts for lignocellulose depolymerisation and intends to determine their conversion rates, distribution on lignocellulose and their interaction.

In the present study we report the heterologous expression and preliminary characterization of an endoglucanase belonging to the CAZy GH45 family from *Formitopsis pinicola*. The recombinant expression plasmid containing FpCel45A was transformed into *P. pastoris* X-33 by electroporation. Zeocin resistant transformants were screened and the best producing colony used for recombinant expression in a methanol-fed batch culture. The enzyme was purified in a two-step procedure using hydrophobic interaction chromatography (Phenyl Sepharose High Performance) and anion exchange chromatography (Qsource15). Two active glycoforms were obtained and verified by electrophoresis and mass spectroscopy. The hydrolysis of various substrates (CMC, PASC and MCC) and the enzymes pH optimum and optimum temperature were determined by the Nelson-Somogyi method and compared to the DNS-assay. The obtained data for this brown-rot GH45 are compared to the previously characterized endoglucanase GH45 from the white-rot fungus *Phanerochaete chrysosporium* [2].

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Mapping the enzyme machineries of cellulolytic soil-dwelling Bacteroidetes

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Keywords: cellulase, cellulose, proteomics, Bacteroidetes.

Bacteria from the phylum Bacteroidetes are regarded as proficient degraders of complex carbohydrates, but most species are limited to soluble glycans, e.g. hemicelluloses and pectins. Two aerobic Bacteroidetes members, *Cytophaga hutchinsonii* and *Sporocytophaga myxococcoides*, have however been known as proficient cellulose metabolizers for decades, but do not conform to the known mechanisms of enzymatic cellulose conversion. Neither species encodes cellobiohydrolases or lytic polysaccharide monooxygenases, and no apparent complexed systems such as cellulosomes have been identified. Many Bacteroidetes species utilize so-called polysaccharide utilization loci (PULs) which encode the necessary enzymes, binding proteins, sugar transporters and regulatory elements for target polysaccharides, but also these are absent in the genomes of *C. hutchinsonii* and *S. myxococcoides*. Mutagenesis studies instead point toward the type IX secretion system being a crucial factor in polysaccharide turnover, and it is also tightly linked to their rapid gliding motility.

In order to shed light on the enigmatic cellulolytic systems of these bacteria, we have used quantitative proteomics to map which proteins they produce during growth on cellulose and pectin, respectively, and determined the proteins' cellular locations [1]. Both bacteria produced similar yet distinct arrays of mostly unstudied putative cellulases during growth, and interestingly, cellulolytic activity was detected not only in the extracellular fraction and outer membrane but also intracellularly. In addition, several glycoside hydrolase family 8 (GH8) enzymes, that have previously been overlooked as potential cellulases in these species, were found to be both abundant and selectively produced during growth on cellulose. These GH8-containing proteins, which comprise large regions of unknown function and range between ~1100-2800 amino acids in total, are currently being functionally characterized to clarify their roles in cellulose turnover.

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Induction of cellulases by cellooligosaccharides in *Fusarium oxysporum* grown in carbohydrate and non-carbohydrate carbon sources

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Keywords: enzyme induction, cellulase, *Fusarium oxysporum*, cellooligosaccharides

Fusarium oxysporum has attracted extensive research interest due to its remarkable ability to produce a wide range of plant cell-wall degrading enzymes [1]. Moreover, the ability of this fungus to simultaneously degrade cellulose and hemicellulose and ferment the sugars to ethanol makes it a great candidate for biotechnological application [2]. Therefore, understanding the factors that induces the production of such enzymes is of great interest. In this study, the influence of cellooligosaccharides of varied chain length and at different concentrations on the induction of cellulolytic enzyme production from *F. oxysporum* was studied. Moreover, we studied the effect of carbon source (sucrose or glycerol) during the growth phase. For the induction studies, washed mycelium produced either on 1% w/w glycerol or 1% w/w sucrose, was suspended in basal medium containing an inducer, i.e. cellobiose (0.1-0.3% w/w), cellotetraose (0.1-0.2% w/w) or cellohexaose (0.1-0.2% w/w) and the induction effect was followed for 3 days. The activity assessment of endoglucanase, exoglucanase, cell-bound and extracellular beta-glucosidase unveiled that the type of carbon source used for the production of the mycelium affected the efficiency of the different inducers for the induction of the different enzymatic activity. Based on the obtained result, the inducer chain length affected the enzyme induction, but this was also affected by the carbon source used for the production of mycelium. More specifically, for cells grown on sucrose, higher molecular weight inducers were more effective. On the contrary, for glycerol-grown cells, low molecular weight cellooligosacharides were more efficient for the induction of cellulolytic enzymes. These data suggest that for *F. oxysporum*, not only the chain length and concentration of inducers is important for enzyme induction, but also the composition of the media and particularly the type of carbon source has an important effect.

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Xyloglucan utilization locus in *Bifidobacterium dentium* bd1

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Keywords: xyloglucan, glucoside hydrolases, proteomics, gut microbiota

The human gastrointestinal tract is colonized by a dense microbial ecosystem responsible for the digestion of complex polysaccharide dietary fibers consumed by humans from a wide range of foods. Xyloglucan is a prominent hemicellulosic polysaccharide constituting up to 25% of common vegetable dry weight [1]. It is composed of β-1,4 linked glucosyl residues heavily substituted by α-1,6 linked xylosyl residues and, depending on the origin, are further decorated by various monosaccharides, including galactose, fucose and arabinose [2]. Xyloglucan utilization has been described for two Gram-negative bacteria, *Bacteroides ovatus* from human gut [3] and the saprophyte *Cellvibrio japonicas* [4].

We have identified a xyloglucan utilization locus (XgUL) in *Bifidobacterium dentium* bd1 that belongs to the *Bifidobacterium* genus, known for its probiotic properties [5]. Interestingly, having been classified as an opportunistic oral pathogen [6], *B.dentium* was one of the most abundant *Bifidobacterium* species in the gut microbiota of centenarians from the renowned Bama region [7]. The *B.dentium* bd1 XgUL is the yet most complex encoding 8 glycoside hydrolases (GHs) belonging to 6 GH families, 6 ABC transporter proteins and an esterase. *B.dentium* grew on xyloglucan as the sole carbon source. Comparative proteomic analysis with label-free quantification identified >40 differentially abundant proteins from 1254 high-confidence protein identifications. The 8 GHs and the esterase were producted recombinantly and purified. The activity on xyloglucan of two endo-xyloglucanases from family GH5_4 was confirmed by TLC and MALDI-MS. The functional characterization of the other GHs is underway and latest results will be presented and discussed.

The molecular investigation of the XgUL of *B.dentium* bd1 will expand our understanding of the sophisticated carbohydrate degradation systems that gut microbes have developed in the competitive gut environment. The gained knowledge will assist the development of strategies to improve human health through applications of probiotics and prebiotics.

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Glycan utilization by butyrate producing bacteria of the healthy gut microbiota

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The human gut microbiota (HGM), has a profound impact on human physiology and health [1]. A pivotal factor that modulates the composition of this community is preferential catabolism of dietary host-non-digestible glycans, which are fermented to various short chain fatty acids (SCFAs). Fecal transplants have unveiled the tremendous potential of manipulating the HGM composition in treatment of metabolic disorders. Insight into the metabolic specialization amongst the HGM, however, offers a more controlled and rational approach to develop future microbiota-based therapeutic interventions. Recently, butyrate producing bacteria of the HGM have attracted particular attention, owing to the key role of butyrate in regulating host gut enterocytes cellular differentiation, apoptosis, gene expression and inflammatory responses [2]. Expectably, levels of butyrate producers inversely correlate with inflammatory disorders e.g. Crohn disease and ulcerative colitis. The majority of these taxa are predicted to be nutritionally highly specialized based on their set of carbohydrate active enzymes (CAZymes) [3]. In contrast to the more intensely studied classical probiotic bacteria from *Lactobacillus* and *Bifidobacterium* [4,5], insight into glycan metabolism of butyrate producers as new probiotic candidates is limited.

Recently, we have demonstrated that members of this taxonomic group are highly competitive during growth on the abundant dietary fibers xylan and mannan [6,7]. Our goal is to further explore differences in glycan utilization profiles and to highlight metabolic specifications of abundant butyrate producers of the healthy HGM. Recent data will be presented to highlight the metabolic niches of individual butyrate producers for limited sets of complex glycans, including those of pectic origin.

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Surface exposed glycosidases of human gut bacteria conferring galactomannan catabolism

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Keywords: gut bacteria, β-mannanase, α-galactosidase, polysaccharide utilization locus.

Galactomannans are hemicellulosic dietary fibers which can be fermented in the gut [1,2]. Here we report on enzyme systems for galactomannan digestion and import among common human gut bacteria, results which contribute to the design of prebiotics.

Gut bacteria may have different synergistic strategies for galactomannan digestion. The studied Bifidobacteria express single surface exposed β-mannanases [3, 4]. *Bacteroides ovatus*, on the other hand, expresses several glycoside hydrolases (GHs) from a polysaccharide utilisation locus (PUL) [5], a gene-cluster which is essential for galactomannan utilization [6,7]. The GHs, two β-mannanases (BoMan26A, BoMan26B) and an α-galactosidase (BoGal36A), act in a sequential manner [6,7]. The two β-mannanases were characterised, including solving the TIM-barrel crystal structures, contributing to a model of the combined function of the enzymes and binding proteins of this galactomannan PUL [7]. BoMan26B is exposed on the cell surface and makes the initial endo-attack on highly branched guar galactomannan, explained by the open and extended active site cleft visible in the recent crystal structure. Oligosaccharide products generated by BoMan26B binds (Kd 4 mM) to a surface-exposed glycan-binding SusD-homolog, predicted to guide import to the periplasm, where BoMan26A acts in synergy with the periplasmic BoGal36A and efficiently releases mainly mannobiose from oligosaccharides.

The narrow active site cleft of BoMan26A and active site loop flexibility, recently studied with ^{1}H, ^{13}C, and ^{15}N NMR, explains the different mode of attack compared to BoMan26B. Phylogenetic analysis place BoMan26A and BoMan26B in different clades of family GH26, from which we can extrapolate a potentially similar set up with two distinct GH26 β-mannanases in PUL-encoded systems among several other Bacteroidetes.

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Mining of the biosynthetic mechanisms of *Vibrio* spp. polysaccharides and potential role in biofilm formation

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Keywords: exopolysaccharide, extrapolymeric substances, biosynthesis, genome

Vibrio spp. are ubiquitous marine bacteria that are ecologically and metabolically diverse members of planktonic and animal associated microbial communities. They encompass the ancient and well-studied human pathogen, *Vibrio cholerae*, and two other human pathogens, *V. vulnificus* and *V. parahaemolyticus*, as well as some less thoroughly characterized animal pathogens. Virulence is based on a wide diversity of mechanisms involved in motility and host colonization, in ability to persist and develop, and in damage generation. Polysaccharides may play major roles in virulence and are major components of extracellular polymeric matrix synthesized upon biofilm growth. They may also exhibit biological features, especially those similar to animal-derived glycosaminoglycans (GAG). Bacterial polysaccharides include EPSs which are released to the surrounding medium, and two surface polysaccharides: lipopolysaccharides (LPS) with an *O*-antigen polysaccharide linked to the Lipid A core complex and capsular polysaccharides (CPS) with K-antigen.

Diversity of biosynthetic pathways involved in glycopolymers biosynthesis in *Vibrio* spp. was analysed through *in silico* identification of genes encoding CAZYmes and comparative genomic approaches. In parallel, ability to form biofilm and extracellular matrix composition is studied. The aim is to provide a better knowledge of the polysaccharide gene cluster importance and to facilitate discovery of new bioactive carbohydrate compounds.

Functional characterization of a FOS transporter system from an uncultured human gut *Dorea* species

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Keywords: Fructo-oligosaccharides (FOS), Microbiome, *Dorea*, Phosphotransferase system (PTS).

Glycosides represent a large fraction of the human diet. Most of them, including prebiotics, are undigestible in the upper part of the gut, and constitute the main energy sources for intestinal bacteria. Prebiotic consumption modulates the gut microbiota composition and functioning, with multiple effects on digestive comfort and human health. For these reasons, the world market for these functional foods is continuously increasing. However, only a handful of the mechanisms of prebiotic utilization by gut bacteria have been established to date, because of the difficulties of addressing the question of oligosaccharide recognition and cellular internalization in native strains, of which the large majority are uncultured.

By using functional metagenomics, we discovered the first pathway of prebiotic metabolization by a non-beneficial gut bacterium. It is encoded by a highly abundant locus in the human gut microbiome, belonging to a dominant uncultured *Dorea* species. It includes a carbohydrate transporting phosphotransferase system (PTS) that we proved, by heterologous expression and locus engineering in *E. coli*, to be involved in fructooligosaccharides (FOS) cellular internalization. We developed a new quantitative approach based on high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), to determine its specificity towards FOS of various polymerization degrees. The functionality of each of the PTS modules was further proved by strain engineering and mass-spectrometry. Finally, integration of biochemical and (meta)genomic data allowed us to revisit the classification and taxonomical distribution of bacterial PTS, and to better understand the interaction between prebiotics and the human gut microbiota.

Characterizing CAZymes from *Chitinophaga pinensis*

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Keywords: *Chitinophaga*, biomass degradation, enzyme discovery and characterization.

We believe that renewable biomass has to be the resource for future materials due to its high sustainability. However, the complexity of biomass makes it challenging to process it using current industrial setups. Enzymes are molecular machines that are able to catalyze specific reactions on complex substrates like plant or fungal cell wall and therefore enzymes are well suited to process biomass. A rich source of biomass modifying enzymes are soil bacteria like *Chitinophaga pinensis* [1, 2]. We have identified a novel glycoside hydrolase in *Chitinophaga pinensis* that is not predicted to be classified as a member of any known glycoside hydrolase family. A characterization of this enzyme will be presented and its potential applications in the processing of plant biomass will be discussed.

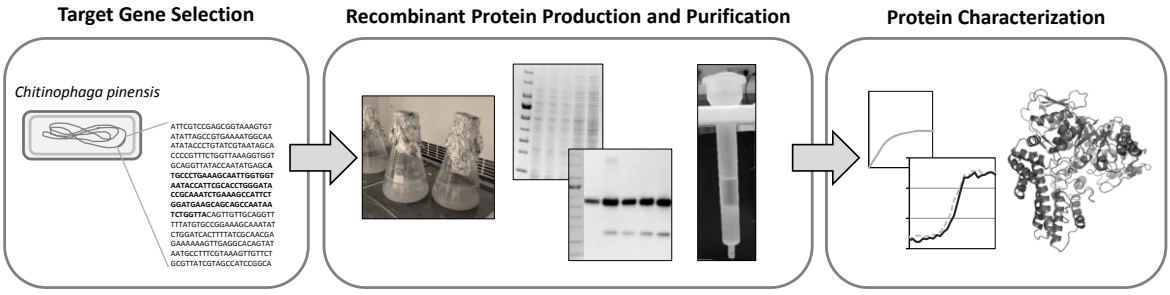

Figure 1. Workflow of characterizing novel carbohydrate active enzymes from *Chitinophaga pinensis*.

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Anaerobic digestion microbiome: a reservoir of carbohydrate active enzymes

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Keywords: Hydrolytic potential, *Bacteroidetes*, Anaerobic digestion, Recombinant protein

The enzymatic hydrolysis of lignocellulosic biomass, i.e. complex polysaccharides, is mainly driven by the action of carbohydrate active enzymes (CAZymes). Therefore, they are key actors in the biotechnological sector. Anaerobic digestion (AD) of biomass, is a process aiming at producing biogas from biomass decomposition, by the joint action of diverse microorganisms. Thus, AD can be seen as a reservoir of CAZymes. Based on our previous study [1], it was shown that the structure of microbial communities was affected by acidosis (volatile fatty acids accumulation), a major dysfunctioning observed in full-scales AD reactors. Interestingly, by applying metagenomics and bioinfomatics to the samples from acidified AD reactors, we showed the functional retention of the microbial hydrolytic potential (driven by the presence of 4148 CAZymes coding genes), even during severe acidosis. Bacterial genomes were re-constructed in order to further link the community hydrolytic potential with key microorganisms. *Bacteroidetes* genomes harbor higher diversity of hydrolytic enzymes, that might favour their dominance over other bacteria in some AD [2-4]. Five CAZymes, belonging to a glucomannan polysaccharide utilization loci (PUL), encoded in the genome of a dominant *Bacteroidetes* sp., were further heterologously expressed in *E. coli*. *In silico* predictions and biochemical characterization enabled us to propose a model of acetylated glucomannan degradation by *Bacteroidetes*.

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Bioprospecting the termite gut for more efficient lingocellulose deconstruction

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Keywords: termite gut system, microbiome, CAZY, *Miscanthus*

Miscanthus is a perennial crop that thrives well in European climate and can be cultivated for 15 to 25 years on marginal lands requiring minimal fertilization and tillage. Its cultivation could offer a new source of revenue to the agricultural sector since *Miscanthus* is regarded as potential bioenergy feedstock, *e.g.* for biogas and bioethanol production [1]. Nevertheless, the high recalcitrance of *Miscanthus* biomass makes it a slowly degradable substrate, difficult to fully valorize in existing biogas plants. Although the biorefinery of biomass to biofuels is a man-made concept, natural organisms/systems can effectively mediate the different steps in the course of the process. Between others, termites developed different lignocellulose digestion strategies that in the case of higher termites mainly depend on the hydrolytic capacities of their gut microbiota. While the currently existing biomass pre-treatment strategies suffer from diverse inherent drawbacks, enzymatic cocktails resembling the complex lignocellulolytic termite system could be an alternative.

In this study, we fed three termite lab colonies of naturally grass-feeding *Nasutitermes* sp. with purely *Miscanthus* diet for a period of ten months. By regularly monitoring the structure of the gut microbial communities using the 16S rRNA gene amplicon sequencing [2], we could clearly notice the microbiome adaptation to the new feed source. Combined metagenomics and metatranscriptomics approach [2], enabled us to unravel novel enzymes and the complexity of lignocellulose degradation by the termite gut system. *Miscanthus* biomass being mainly composed of cellulose (40-50 %), hemicelluloses (25-30 %, including mainly arabinoxylan, β-glucan and xyloglucan) and lignin (10 %), triggered up regulation of specific clusters of carbohydrate active enzymes (CAZY). While the host seemed mainly involved in cellulose and to a lesser extent lignin digestion (putative laccase was discovered in the termite transcriptome), its gut microbes targeted mainly hemicelluloses (*Spirochaetes*) and cellulose (mainly *Fibrobacteres* and some *Spirochaetes*). Interestingly, multiple clusters of co-localized CAZY genes resembling polysaccharide utilization loci (PULs) of *Bacteroidetes*, targeting both cellulose and different hemicelluloses, were discovered in re-assembled genomes of novel *Spirochaetes*. In continuation, based on predicted CAZY activities and gene expression profiles, selected CAZY genes and whole CAZY clusters of both termite and microbial origin, were cloned and over-expressed in specific hosts. We hope that combining these enzymes in enzymatic cocktails will soon help us to over-come the recalcitrance of *Miscanthus* biomass.

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Fungal biodiversity as a source of enzymes with great biotechnological potential

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Keywords: Filamentous fungi, biodiversity, CAZyme, lignocellulosic biomass, biorefinery.

Filamentous fungi are among the most potent degraders of lignocellulosic biomass due to their ability to thrive in lignocellulose-rich environments; they produce a high number and a broad variety of enzymes that have different and complementary catalytic activities. A large screening of the fungal CIRM-CF collection (http://cirm.esil.univ-mrs.fr/) using robotic methods specifically developed in-house led to the identification of filamentous fungi that deconstruct efficiently polysaccharides. These investigations enabled the unbiased identification of fungal strains issued from biodiversity with high biotechnological potential. To understand their mechanism of action, some of these fungal strains were further investigated using state-of-the-art genomic, transcriptomic and proteomic techniques. The comparative analysis of fungal secretomes (the proteins present in the culture supernatant) using advanced proteomics highlighted the cooperation between fungal enzymes for enhanced degradation of complex lignocellulosic substrates and some discrepancies in carbohydrate-active enzymes (CAZymes) sets dedicated to different types of biomass. For instance, we evidenced that the fast solubilization of recalcitrant cellulosic biomass by the basidiomycete fungus *Laetisaria arvalis* involved successive secretion of oxidative and hydrolytic enzymes.

Over the last ten years, hundreds of fungal secretomes have been analyzed. Each secretome contains an average number of 100 enzymes per secretome for a given growth condition, which means that several thousands of lignocellulose-acting enzymes have been identified. To manage these data, an internal database (ProteoDB) gathering all the fungal secretomes obtained until now has been developed and allows comparative analyses and identification of enzymes of interest with a special attention given to CAZymes.

Capturing biochemical information in the CAZy database

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Keywords: CAZy; enzyme function; community service.

Since 1998, the Carbohydrate-Active enZYmes (CAZy; www.cazy.org) [1] database describes the sequence-based families of enzymes and associated domains that cleave, modify, and build glycans. As of today CAZy lists more than 1.2 million entries arranged in over 320 enzyme families, including data from over 13,300 genomes. This classification provides a system that correlates structural features and molecular mechanism of carbohydrate active enzymes and has become a reference system in our field.

Our expert biocuration of sequence and functional information provides the community a manually curated and up-to-date knowledge. Within the CAZy system, enzyme specificity is displayed (in the form of an EC number) only based on experimental evidence. The assignment of a function to an entry thus depends on the availability of experimental data, ideally providing substrate (and/or product) specificity. The exponential growth of sequence data requires extensive automation for the identification of CAZymes. After more than 20 years of continuous activity, the classification and modular analysis of these enzymes has reached maturity. However, the management of functional data still relies on limited vocabularies and on heavy biocuration.

In order to pursue this effort and to make functional biocuration data available to the entire community, we have implemented on our website a form to submit the minimum information required to assign a function to a sequence. We reasoned that this process would be less time consuming and less error-prone if the researcher at the origin of a newly characterized sequence provides these details. Thus with the help of our community, we will be able to dedicate our efforts to improving our database and associated tools to cope with the ever-increasing amount of data and contribute a better understanding of the metabolism of complex carbohydrates in general.

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Selective labeling of carbohydrate-binding proteins by triazinyl-glycosides

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Keywords: Oligosaccharide-based probes, Protein labeling, FRET, Click chemistry

With the availability of complete genome sequences for numerous prokaryotic and eukaryotic organisms, the number of novel genes in need of functional assignment increases dramatically. Chemical probes for selective covalent labeling of proteins are thus of great interest for the isolation of biological receptors or functional characterization of putative enzymes [1]. Photoaffinity-based probes and mechanism-based inhibitors are among the most investigated means to isolate target proteins. However, because of the difficulties associated with the synthesis and manipulation of oligosaccharides, most of the examples reported to date concern mono-and di-saccharide derivatives.

In the present work, we report a novel class of carbohydrate probes i.e oligosaccharidic triazinyl glycosides allowing the selective labeling of lectins and CAZymes. Triazinyl glycosides can be efficiently obtained from unprotected carbohydrates in aqueous media [2]; they can also be chemically modified with two bioorthogonal reporters [3]. As an illustration, we will describe the synthesis of chitinpentaose-based probes and their evaluation against Wheat Germ Agglutinin, *Bacillus circulans* chitinase A1 and Hen Egg White Lysozyme. In particular, an activity-based probe allowing both continuous fluorescence detection of chitinase activity by FRET and protein labeling will be presented.

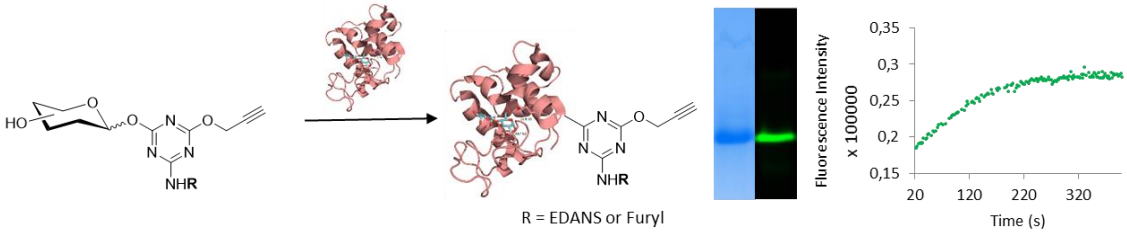

Figure 1. Selective labeling of carbohydrate binding proteins by triazinyl-glycosides.

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High-throughput substrate specificity analysis of metagenomic-derived arabinoxylan-active enzymes

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Keywords: metagenomics, enzyme discovery, substrate specificity, DSA-FACE.

Functional enzyme screening of the increasing amount of metagenomic data is often cumbersome, especially for the discovery of enzymes with complex substrate specificities such as carbohydrate active enzymes. We present our high-throughput approach based on DNA sequencer-aided fluorophore-assisted carbohydrate electrophoresis (DSA-FACE) [1] for the parallel analysis of carbohydrate specificities of putative carbohydrate active enzymes present in metagenomics data.

A metagenomic study on the North American beaver (*Castor canadensis*) feces revealed the presence of putative arabinoxylan-active enzymes from uncharacterized subfamilies 2, 7 and 28 of the GH43 CAZy family and of the GH8 family [2]. To study the substrate specificity of these newly-discovered enzymes, twelve arabinoxylan-oligosaccharides were evaluated as substrates for different enzyme concentrations. DSA-FACE analyses showed unprecedented endo-β-xylanase, β-xylosidase, reducing end xylose-releasing exo-oligoxylanase (Rex) and α-L-arabinofuranosidase activities, including quantitative substrate preferences. These analyses give insight in new arabinoxylan degradation patterns which have potential application in the biorefinery industry.

The generic nature of our approach allows the detection of many carbohydrate active enzymes, including their specificity. In addition, the method allows to use natural substrates instead of synthetic substrates such as fluorogenic and chromogenic compounds, which may mask the natural enzyme specificity. This is essential for a more accurate assignment of the specificities of newly-discovered enzymes.

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CAZyme organization and utilisation in the soil Bacteroidetes *Chitinophaga pinensis*

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Keywords: *Chitinophaga*, Bacteroidetes, Polysaccharide Utilisation Loci, microbial cell wall.

Using a combined approach of classical microbiology with biochemical enzyme assays, carbohydrate structural analysis, proteomic mass spectrometry, and genome sequence analysis, we have performed extensive investigations into the soil Bacteroidetes *Chitinophaga pinensis*. The genome of this species encodes at least 191 glycoside hydrolases from 56 different families, and the predicted activities of these enzymes show the potential for degradation of a wide variety of glycans. However, we have shown that the species has a strong metabolic focus on β-glucans derived from microbial biomass [1,2]. Many of the predicted chitinase and β-glucanase enzymes that we predicted are used to deconstruct fungal cell walls are secreted via the recently described Type IX Secretion System, and many are large multi-modular proteins.

Ongoing projects are aiming to characterise enzymes from the fungal cell wall-targeting apparatus of *C. pinensis*, and this poster will give an update on the characterisation of some interesting target proteins.

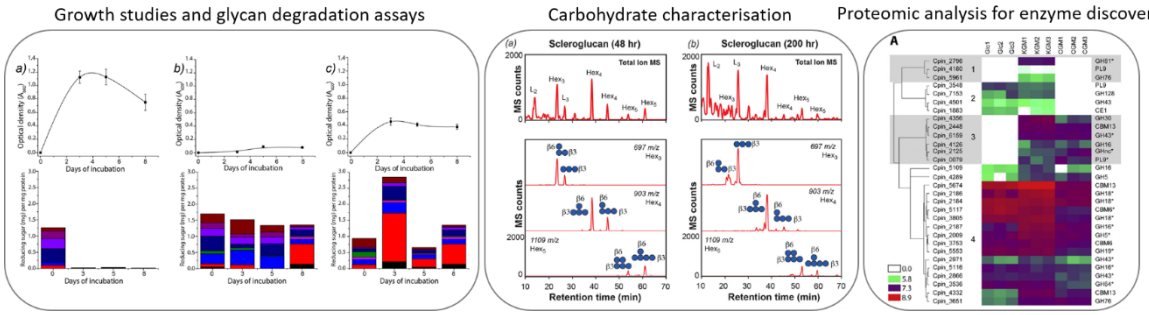

Figure 1. We have used a combination of bacterial growth studies, enzyme assays, carbohydrate deconstruction analysis and proteomic investigation to uncover enzyme targets for characterisation.

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Novel cazymes from microbial communities populating geothermal environments for biotechnological applications.

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Keywords: metagenomics, hyperthermophiles, cazymes, glycosidases.

Novel thermophilic glycosidases, showing uncommon intrinsic stability to pH extremes and temperatures >80°C[1] are very promising candidates for the biotransformations and biotechnological application requiring extreme reaction condition as for lignocellulosic materials in second-generation biorefineries. We report here a metagenomic approach aimed to search novel Cazymes[2] within the hyperthermophilic microbial communities populating geothermal sites. The metagenomic analysis of the microbial consortia in two neighboring mud/water pools in the solfataric field of Pisciarelli (Naples, Italy) that differ in temperature and pH (Pool1 T=85°C and pH 5.5; Pool2 T=94°C and pH 1.5) was performed. Moreover, to identify enzymes to be exploited in the conversion of lignocellulosic biomasses for second-generation biofuels, we enriched in-lab Pool1 community to select microorganisms able to grow on different plant biomasses.
The analysis of metagenomic data revealed a high abundance of cazymes in the solfataric samples. In particular, within the cazymes present in Pool2, we identified and characterized a novel hyperthermostable GH5 β–mannosidase and the first archaeal GH109 showing activity on glucosides and *N*-acetyl-glucosides.
In addition, among the sample enriched in-lab on plant biomasses, a remarkable selection of specific cazyme families was observed and the characterization of a set of these enzymes revealed novel α-glucosidase and glucanase activities.
We show here that a combined approach of metagenomic of extreme environments, in-lab enrichments, and detailed enzymatic characterization is a powerful tool to exploit natural biodiversity and obtain novel biocatalysts for industrial applications.

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Development of new functional metagenomic approaches to investigate the host-microbiome cross-talk

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Keywords: Functional metagenomics, droplet-microfluidics, human gut microbiota, mucin-oligosaccharides.

The human gut microbiota is essential for maintaining host health. Imbalanced gut microbiota is associated with an alteration of the mucus layer firewall which lines the intestinal epithelium, and with inflammatory bowel diseases. However, the molecular bases of the interactions between the host and gut microbes, especially the non-cultivated ones, remain under-investigated. In order to better understand the enzymatic synergies naturally displayed by gut bacteria to degrade host glycans, we designed a new functional metagenomic approach based on droplet microfluidics. Speeding up enzyme discovery and complementing previously established conventional screening methods, it is compatible with fosmid libraries, facilitating the isolation of not only isolated enzymes but also of complete catabolic pathways. The functional characterization of novel enzymes capable of breaking down host glycans paves the way for deciphering the microbiota-host crosstalk and, to a further extend, for the control of their interactions in pathological contexts.

Acknowledgments
This project has received funding from the INRA metaprogramme M2E (project Metascreen) and from the European Union's Horizon 2020 research and innovation programmes under grant agreements n° 685474 (project Metafluidics) and n°707457 (project CatSYS).

Unraveling linear scaling relations for cellulases

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Keywords: cellulases, linear scaling relations, catalytic rate constant, affinity

Historically, the principles and methods from non-biochemical catalysis have been poorly explored in the context of enzymology. A recent breakthrough, however, has developed a new principle that correlates activity and affinity of interfacial enzymes *via* re-interpretation of kinetics parameters[1]. The method combines a classic principle from (non-biochemical) heterogeneous catalysis – the Sabatier Principle – with the fundamental principles of enzymology in order to verify its applicability on heterogeneous biochemical catalysis. In this work, we expand the application of such practices to interfacial enzyme catalysis, specifically the concept of linear free energy relationships (LFERs) or linear scaling relations – which are connected to the Sabatier Principle. In order to do so, kinetic parameters were determined against microcrystalline cellulose (Avicel) *via* Michaelis-Menten approach for dozens of cellulases from 6 species, including 8 wild-types. The enzyme set was formed mostly by CBHs and EGs possessing different catalytic mechanisms and structures. Then, these parameters were plotted against each other in order to find correlations between the transition energy of the slowest catalytic step $\Delta G^{\ddagger}$ and enzyme-substrate binding energy ΔG^{o}_{B} – or simply k_{cat} and K_M. Once a strong correlation (R>97%) was found, published data for cellulases, obtained by different individuals in our group, using the same substrate and similar experimental conditions, was merged into a single graph. The effectively conserved linear relation of $\log_{10}k_{cat}$ and $\log_{10}K_M$ (representative of $\Delta G^{\ddagger}$ and ΔG^{o}_{B}) shows, for the first time to our knowledge, the existence of linear scaling relations across a large variety of interfacial enzymes – specifically cellulases. The impressive fact that a simple rule can predict catalytic rates based on affinity (and vice versa), for different structures spread in nature, opens up new possibilities for the understanding of the physical limitations of cellulase activity and how this affects the natural selection of these enzymes.

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Enzymatic degradation of semidilute hemicellulose solutions: A small-angle neutron scattering study

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Keywords: SANS, arabinoxylan, xylanase, arabinofuranosidase

When they penetrate and degrade a lignocellulosic material, enzymes work in conditions where the local concentration of polymer(s) may be elevated. In such situations, it is still not clear how the spatial constraints experienced by the enzymes potentially affect their kinetics and the nature of the products released, i.e. short versus long polymer chains depending on the balance between enzymes diffusion and reaction.

Here we explore this question by using a 'more physical than biological' approach based on the use of small-angle neutron scattering (SANS), a powerful technique that gives access to structural information at length scales ~0.5-500 nm. The substrates are model solutions of arabinoxylan (AX) at concentrations ranging from 1-150 g/L. As illustrated in the next figure, we have strong and clear effects of the enzymatic action on the SANS intensity. In our poster contribution, we propose to discuss these changes in relation with the two types of enzymes used (Xyn vs Abf, see Fig. 1), their actual activity (number of cleavages performed), and the AX concentration in the solutions.

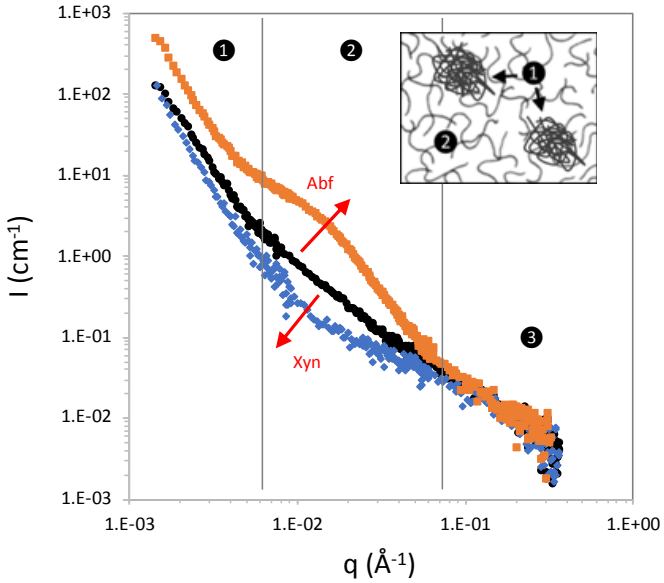

Figure 1. Neutron scattered intensity as a function of scattering vector for a ~50 g/L arabinoxylan solution before (black circles) and after ~24 h of the action of a xylanase (blue diamonds, Xyn arrow) and an arabinofuranosidase (orange squares, Abf arrow) at 1 and 4 µM, resp. At low q (regions ❶, the intensity originates from large associated structure while in regions ❸the intensity results from the local conformation of the chains. In regions ❷ intermediate scales are explored.

Screening a site-saturation mutagenesis library of a fungal chitin deacetylase to probe the substrate binding site

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Keywords: chitin deacetylase, site-saturation mutagenesis library, screening assay

Chitosans are functional biopolymers, consisting of *N*-acetyl-*D*-glucosamine and *D*-glucosamine units. They are mainly characterized by their degree of polymerization (DP) and their degree of acetylation (DA), but also their pattern of acetylation (PA). Chitosans are commercially derived from chitin by deacetylation using sodium hydroxide, where the DA can be controlled, whereas the PA remains random when using chemical methods. Therefore, chitin deacetylases (CDAs) catalyzing regio-selective deacetylation of chitinous substrates can be used to generate chitosans with defined, non-random PA.

In recent years, a growing number of CDAs generating different defined partially acetylated chito-oligosaccharides (paCOS) have been described. However, the mechanism underlying the regio-selectivity of the enzymes that eventually result in the production of different paCOS are poorly understood. Based on the crystal structure of VcCDA from *Vibrio cholerae*, Andrés et al. proposed the subsite-capping model to explain the observed binding of small chitin oligomers (DP 2-3) in the active site of the CDA enzymes by different conformations of six loop regions surrounding the binding site [1]. In contrast to VcCDA, crystal structures of other CDAs exhibit a more open binding site [2, 3].

To gain a better understanding of the substrate binding in CDAs with a more open binding site, a site-saturation mutagenesis library for the fungal CDA from *Pestalotiopsis sp.* (PesCDA) [4] was generated covering 27 amino acids surrounding the active site. The muteins where tested towards their activity on chitin tetramer (A4) using a high-throughput screening developed for VcCDA [5]. The exchange of these amino acids against almost all non-wildtype amino acids allowed more in-depths conclusions regarding the function of each residue. Thus, the results allowed us to identify amino acids which are crucial for substrate binding and, therefore, enzyme activity. In further screenings, different defined paCOS will be tested as a substrate for the different muteins to get further insights into the substrate binding of CDAs that defines the regio-selectivity of the enzymes and, therefore, the generation of different PAs by different CDAs.

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Crystallization, characterization and mechanistic analysis of a novel UDP-glucuronic acid 4-epimerase

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Keywords: UDP-D-galacturonic acid, SDR enzyme, epimerase, kinetic isotope effect

UDP-glucuronic acid 4-epimerases catalyze NAD^+ dependent interconversion of UDP-D-glucuronic acid (UDP-GlcA) and UDP-D-galacturonic acid (UDP-GalA), both essential precursors for the synthesis of cell-wall polysaccharides in plants and bacteria (Fig.1) [1].

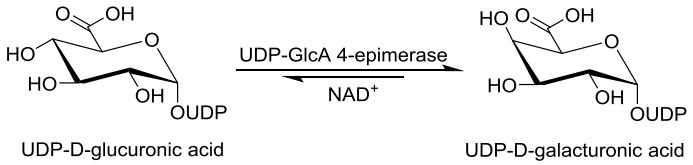

Figure 1. NAD^+ dependent epimerization of UDP-GlcA to UDP-GalA catalyzed by UDP-GlcA 4-epimerase.

The catalytic mechanism of UDP-GlcA 4-epimerases has not been solved. Nonetheless, the comparison to other short-chain dehydrogenase/reductase (SDR) proteins, e.g. UDP-galactose 4-epimerases, suggested a reaction via formation of a β-keto acid intermediate [2]. We are in particular interested how UDP-GlcA 4-epimerases, other than UDP-D-xylose synthase (UXS) or UDP-D-apiose/D-xylose synthase (UAXS) [3,4], prevent decarboxylation of the rather instable reaction intermediate. Here, we show the crystallization and biochemical characterization of a novel UDP-GlcA 4-epimerase (BcGlc-epi2) from *Bacillus cereus HuA2-4*. The crystal structure of BcGlc-epi2 was solved in Michaelis-Menten complex with its natural substrate UDP-GlcA and cofactor NAD^+ at 1.3 Å resolution. The crystallographic information combined with kinetic isotope effect and mutagenesis studies confirmed a reaction mechanism that proceeds via a transient ß-keto acid intermediate. Moreover, key amino acid residues important for preventing substrate decarboxylation could be identified. In order to gain a more detailed understanding on the epimerase kinetics and back reaction, we synthesized the commercially not-accessible product UDP-GalA using a novel one-pot enzymatic cascade. UDP-GalA has proven to be a valuable mechanistic probe for crystallography and kinetic studies, allowing novel insights into the reaction from UDP-GalA to UDP-GlcA.

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From raw starch degrading *Bacillus paralicheniformis* α-amylase to transglycosylase by single point mutation

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Keywords: α-amylase, transglycosylase, raw starch, mutation.

Understanding the structural peculiarities and properties of starch as a substrate but at the same time understanding the structural features of raw-starch-digesting amylases (RSDA) is needed for efficient application in health and other branches of industry. Although knowledge of these structures and properties has increased significantly in recent years, it is still a riddle what is the major requirement for RSDA to be efficient in raw starch hydrolysis.

a-Amylase from *Bacillus paralicheniformis* ATCC 9945a (*Bli*Amy), a potent enzyme for raw starch hydrolysis, is an enzyme lacking starch-binding domain. We have recently identified the SBS of *Bli*Amy by crystallographic study of its native form and in complexes with maltose, acarbose, maltohexaose and β-cyclodextrin. Obtained results show that all tested ligands bind at same position to *Bli*Amy. The role of the SBS has been studied by alanization of the identified key residues.

RSDA can be further exploited for its robustness by altering its activity and converting this hydrolase into transglycosylase. The use of transglycosylases for synthetic purposes has been limited since these enzymes are relatively rare in nature. Furthermore, the ones that have been characterized act on a limited substrate repertoire. By contrast, hydrolases are extremely common and act on a wide range of substrates. To alter the activity of *Bli*Amy, His235 was replaced with Glu. The mutant enzyme, H235E, was characterized in terms of its mode of action using different substrate (starch, amylopectin, maltooligosaccharides etc.). H235E exhibited high transglycosylation activity, while the wild-type *Bli*Amy exhibited high hydrolysis activity exclusively. Converting hyperthermostable *Bli*Amy into transglycosylase yields a highly potent tool in synthesis of the starch derivatives.

Acknowledgements

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Multidisciplinary Approaches for the Study of Structure-Activity Relationships of GH70 Glucansucrases

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Keywords: glucansucrases, dextran, structure-activity, X-ray crystallography

Glucansucrases belonging to the GH70 Family (CAZY) represent an important class of enzymes that are widely used for biotechnology and industrial applications. Glucansucrases are able to catalyze the polymerization of glucose units forming a wide range of α-glucan polymers using sucrose, an abundant and inexpensive agro resource, as the sole substrate. Therefore, the understanding of structure-activity relationships of GH70s is essential for the development of tailor-made enzymes able to synthesize polysaccharides or oligosaccharides with precise chemical structure and molecular weight. Glucansucrases are difficult-to-study enzymes, given their high molecular weight (100-200 kDa), their multi-domain organization with intrinsic flexibility and the complexity of the products formed. In the present work, we have applied a combination of multidisciplinary techniques to structurally and functionally characterize the DSR-M enzyme, an efficient dextransucrase that is able to synthetize very linear dextrans with high specificity (99% of α-1,6 linkages).[1] The crystal structure of DSR-M has been solved in complex with substrates and acceptor molecules which revealed the important residues and the structural features that play a role in the catalysis.[2] Small angle X-ray scattering confirmed the *horseshoe* structure of DSR-M which shows a unique glucan binding domain (GBD) that plays a role into dextran binding and elongation. Nuclear magnetic resonance on the ^{15}N labeled enzyme has been used to get insights into the active site dynamics of DSR-M and to characterize one interesting mutant (Trp624Ala) which shows a product distribution shifted to the formation of shorter dextran chains. Finally, using a Monte Carlo simulation of the elongation process, we shed new lights on the mechanistic behavior of sugar polymerases, explaining how the probability of enzyme-acceptor encounters directly affects the product distribution length.

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Oxazoline or oxazolinium ion? The reaction mechanism of GH18 chitinases

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Keywords: glycosidases, molecular modeling, conformational analysis, reaction mechanisms

Chitinases are glycosidases responsible for cleaving the glycosidic bond of chitin, one of the most abundant carbohydrates in nature. These enzymes have gained interest because of their medical and industrial applications.[1] Family GH18 chitinases, unlike most retaining glycosidases,[2] follow a substrate-assisted mechanism in which the 2-acetamido group of the *N*-acetylglucosamine located at the -1 subsite reacts with the sugar anomeric carbon, forming an intermediate which is commonly described as an oxazolinium ion.[3,4] By means of QM/MM metadynamics simulations on chitinase B from *Serratia marcescens*, we analyzed the entire reaction mechanism, showing that the reaction intermediate features a neutral oxazoline, with an oxazolinium ion being formed only *on the pathway* toward the reaction products. The role of a well-defined hydrogen bond network that orchestrates catalysis by protonation events, is discussed.[5]

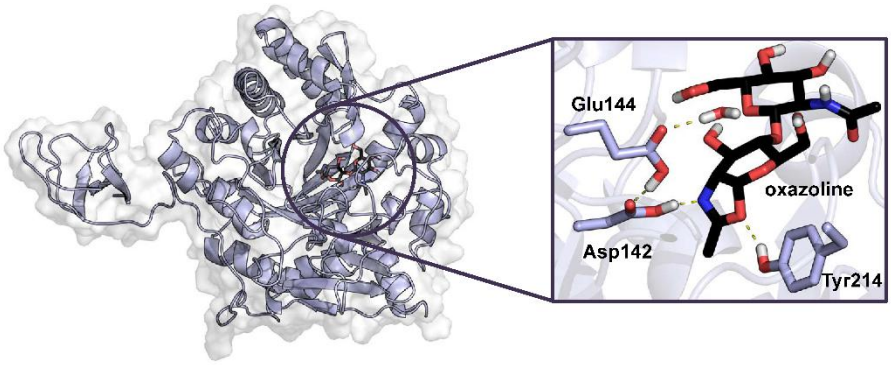

Figure 1. Reaction intermediate of GH18 chitinase B from *Serratia marcescens* obtained from QM/MM simulations.

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α-1,6 mannosidase generates N-glycan specificity through requirement for GlcNac at the +2 subsite.

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Keywords: Glycoside Family 92, alpha mannosidase, N-glycan, *Bacteroides thetaiotaomicron*

In the human gut microbiota *Bacteroides thetaiotaomicron* adopts a glycan generalist strategy to utilise the vast array of indigestible dietary and host polysaccharides as a carbon source. While many of the Polysaccharide Utilisation Loci (PULs), which target dietary glycans, have been characterised, less is understood about N-glycan utilisation. High mannose N-glycans (HMNG) are abundant in the gut from host proteins and glycosylated fungal proteins. The core pentasccharide of HMNG provides the basic structure of other N-glycans such as complex, hybrid and plant N-glycans. *B. thetaiotaomicron* has a dedicated HMNG PUL consisting of an endo-β-N-acetylglucosaminidase, glycoside hydrolase (GH) family 18, localiased to the cell surface which removes the N-glycan from the protein. The glycan is transported in to the periplasm, where a number of exo-acting α-mannosidases, from family GH92, further digest the glycan from $Man_9GlcNAc$ to leave a trisaccharide of Man-α1,6-Man-β1,4-GlcNac [1]. The HMNG PUL does not encode the enzymes necessary to degrade this trisaccharide, however, a further GH92 and a GH130 are encoded in a distinct operon which is expressed at a high basal levels. Here, we describe the biochemical and structural characterisation of an GH92, α-1,6 mannosidase from this operon which requires GlcNac in the +2 subsite of the active site. We present kinetics against a range of N-glycan substrates, the Apo structure of the enzyme and kinetics for mutants in the putative GlcNac binding site. The requirement of the +2 subsite to be occupied is unique for GH92 enzymes and suggests this enzyme has a specific role in processing of the Man-α1,6-Man-β1,4-GlcNac product. This N-glycan specific GH92 is required for complete degradation of all N-glycan substrates degraded by *B. thetaiotaomicron*, which share the core pentasaccharide N-glycan structure.

Inhibition of sialidases from pathogens

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Keywords: sialidase, inhibition, thiosialoside, multivalency.

Sialidases (SAs) hydrolyze sialyl residues from glycoconjugates of the eukaryotic cell surface and are virulence factors expressed by pathogenic bacteria, viruses, and parasites. Effective inhibitors were developed for virus SAs (zanamivir and oseltamivir are nanomolar inhibitors of *influenza* SA) but are poor inhibitors of SAs from bacteria and parasites, for which literature only reports molecules active in the micromolar range for the best cases. There is therefore real opportunity for the development of more potent and selective inhibitors of parasitic and bacterial SA. Contrary to human SAs, the catalytic domains of bacterial SAs are most often flanked with carbohydrate-binding modules or lectin-like domains previously shown to bind sialosides and to enhance enzymatic catalytic efficiency [1]. This structural peculiarity has been poorly studied and has not yet been exploited to design efficient and specific bacterial SAs inhibitors. We aim at designing a new family of sialidase inhibitors that are able to interact with these catalytic and lectinic(s) site(s), simultaneously.

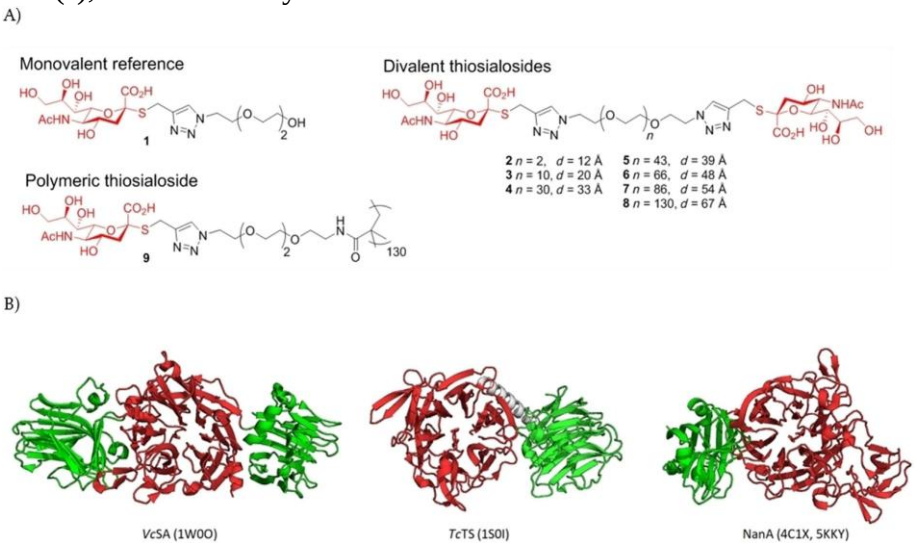

Figure 1. A) Structures of synthetic thiosialosides. B) Structures of the SA targets.

Non-hydrolyzable multivalent thiosialosides were designed as probes and inhibitors of *V. cholerae*, *T. cruzi* and *S. pneumoniae* (NanA) sialidases. NanA was truncated from the catalytic or lectinic domains to probe their respective roles upon interacting with sialylated surfaces and the synthetically designed di- and polymeric thiosialosides [2].

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RHA-P: Structural and functional insight into a novel bacterial α-L-rhamnosidase from *Novosphingobium* sp. PP1Y

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Keywords: Rhamnosidase, Flavonoid, Homology modeling, Novosphingobium sp. PP1Y.

α-L-Rhamnosidases (α-RHAs, EC 3.2.1.40) are glycosyl hydrolases (GHs) hydrolyzing terminal α-L-rhamnose residues from different substrates such as heteropolysaccharides, glycosylated proteins and natural flavonoids. Although the possibility to hydrolyze rhamnose from natural flavonoids has boosted the use of these enzymes in industry, to date only few bacterial rhamnosidases have been fully characterized and only one crystal structure of a α-RHA of the GH106 family has been solved [1]. A novel α-RHA (RHA-P) activity was identified in the crude extract of *Novosphingobium* sp. PP1Y [2]; this enzyme is an *inverting* GH, for which an initial biochemical characterization has been performed [3]. In this work, we show that the enzyme, whose recombinant expression in *E.coli* and purification was optimized, has a good stability in various conditions of temperature and pH, which is ideal for biotechnological applications. An initial homology modeling study of RHA-P, in combination with a site directed mutagenesis analysis, confirmed a pivotal role of conserved residues D503, E506, E644, likely located at the catalytic site and the possible role of an essential calcium ion for catalysis. The definition of the 3D structure through X-ray crystallography is currently underway and will give important details concerning the the catalytic mechanism of this protein and the molecular determinants responsible for it. In addition, RHA-P showed activity on natural flavonoids such as naringin, rutin, hesperidin and quercitrin, with a catalytic efficiency comparable or even higher than other bacterial α-RHAs described in literature. These results suggest that RHA-P have a higher hydrolyzing capacity and may locate different polyphenolic aromatic moieties in the active site, making this enzyme appealing for the bioconversion and de-rhamnosylation of natural flavonoids. In this framework, the possibility to use as biocatalyst, according to the needs, either the purified enzyme or the recombinant whole cell of *E.coli* expressing the protein, render RHA-P a versatile tool for biotechnological purposes.

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Investigating the multimodularity of a Xyn10C-like protein found in a termite gut

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Keywords: protein domain insertion, xylanase, CBM4 tandem, PUL.

In the context of bioeconomy, the discovery and study of plant-cell wall degrading enzymes is particularly relevant for the use of lignocellulosic biomass for industrial purposes. In this respect, functional metagenomics has proven to be a powerful tool to discover new enzymes from a variety of microbial ecosystems, as exemplified by the functional screening performed on the gut of the termite *Pseudacanthotermes militaris* [1]. This study revealed an interesting hypothetical xylan utilization system, encoding five glycoside hydrolases (GH) and one carbohydrate esterase (CE) annotated from Bacteroidales. Among the GHs, a novel GH10 encoding sequence, *Pm*25 showed details of an unusual domain organization (Figure 1). It consists of one catalytic domain, which is intercalated by two Carbohydrate Binding Modules (CBMs). A homologue of this enzyme sharing the same architecture, Xyn10C, was vastly distributed in different xylan utilization loci found in gut Bacteroidetes, which indicated its importance in the glycan foraging for the gut microbiota [2].

Figure 1: Scheme of the *Pm*25 multimodular organization

In an effort to understand its unusual multi-modularity, detailed biochemical and structural characterization of *Pm*25, with or without the CBMs was performed. The role of the CBMs was also investigated and quantification of their interactions towards carbohydrate [3] lead to better understand the specific role of these modules. Overall this study highlighted the important role of *Pm*25 homologs in the xylan utilization system in Bacteroidetes, and pinpointed the meaning of its unusual architecture.

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Combining multi-scale modelling methods to decipher molecular motions of a Branching Sucrase from GH Family 70

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Keywords: ΔN_{123}-GBD-CD2, glucansucrase, MD, NMA, carbohydrates, multi-scale modelling, enzyme sequence-structure-dynamics relationships.

Glucansucrases from Glycoside-Hydrolase Family 70 are valued tools in chemistry to generate glycodiversification. Of great biotechnological interest, these enzymes use sucrose, a very cheap and abundant agro-resource, to catalyze the synthesis of a range of carbohydrates. Here, our study was focused on the first engineered enzyme, called ΔN_{123}-GBD-CD2, which is specific of the α-(1→2) branching of dextran, a rare and non-digestible linkage. Although its original U-shape three-dimensional organization has been recently established, the detailed investigation and description of the structural organization and the functional role of macromolecular motions of these multi-domain enzymes are still missing to provide a comprehensive understanding of the enzyme reaction.

By combining long molecular dynamics simulation (1µs) and multiple analyses (NMA, PCA, Morelet Continuous Wavelet Transform and Cross Correlations Dynamics), we investigated here the dynamics of ΔN_{123}-GBD-CD2 alone and in interaction with sucrose substrate. Overall, our results provide the detailed picture at atomic level of the hierarchy of motions occurring along different timescales and how they are correlated, in agreement with experimental structural data. The findings of our *in silico* study might offer a novel insight on key regions and amino acid residues that could be targeted to design enzyme variants endowed with improved properties for biotechnological applications.

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Study of the catalytic mechanism of PMTub, a new target for the research of innovative molecules active on *Mycobacterium Tuberculosis*.

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Keywords: Protein-*O*-mannosyltransferase, type-C glycosyltransferase, phenotypic bioassay, catalytic mechanism.

Tuberculosis is one of the top 10 causes of deaths (1,6 million in 2017) and the leading cause from a single infectious agent, *Mycobacterium tuberculosis* (Mtb). Today, the drug-targeting of non-essential mycobacterial virulence factors appears to be a promising avenue for reducing Mtb pathogenicity without inducing selection pressure responsible for antimicrobial resistance.
In this context, we have demonstrated that the mannosylation of mycobacterial proteins, catalyzed by the protein-*O*-mannosyltarnsferase PMTub, is not essential for bacterial growth, but crucial for virulence [1]. This enzyme constitutes thus an attractive target for the development of new anti-virulence drugs.

PMTub is a 13-segment transmembrane protein that transfers a unit of mannose from a phospholipid to a secreted protein. Its topology and properties of interface biocatalyst constitute real challenges for the reliable and handy measurement of its activity. Its targeting by putative antagonists is another difficulty resulting from the necessity for these molecules to cross the mycobacterial cell wall, and then to inhibit very specifically the bacterial enzyme without affecting the physiology of the infected host.
In order to fulfil these demanding prerequisites, we have developed a phenotypic bioassay to monitor PMTub activity, and modelled a 3D structure by homology with type-C glycosyltransferases. Preliminary structure-function data allowed us to validate the essentiality of certain amino acids (either conserved in eukaryotes, or mycobacteria-specific) and to propose for the first time a catalytic mechanism for the CAZY GT39 enzyme family.
This preliminary, indispensable basic research work paves the way for semi-rational design of PMTub inhibitors that could lead to the implementation of new therapeutic strategies against tuberculosis.

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Using alginate epimerases as a basis to understand polymer and protein design

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Keywords: epimerases, alginate, CAZymes, ITC

Alginate is a linear anionic polysaccharide composed of β-D-mannuronate and α-L-guluronate and it has a wide range of industrial, medical and pharmaceutical applications. The bacterium *Azotobacter vinelandii* encodes a family of seven secreted and calcium ion-dependent mannuronan C-5 epimerases (AlgE1-AlgE7) that convert on the polymer level mannuronate residues (M) to guluronate residues (G) in the alginate chain. They do so in a processive manner, creating block structures of G- or alternating MG-residues.

Rational design of these alginate epimerases can make it possible to produce enzymes efficient at making tailor-made alginate for diverse applications, with specific compositions of M and G. This requires a better functional understanding of the enzymes mechanisms and mode of action than what we have today.

To acquire this we are using a combination of computational and experimental methods to study the epimerase AlgE4 from *A. vinelandii*. Several mutants are expressed so far, and their activities and binding strengths have been measured with spectrophotometric activity assay, NMR and ITC. The studies point to the importance of substrate binding in regulation of enzymatic activity, and charged amino acids are essential in binding of the polyanionic substrate. Both positively and negatively charged amino acids in the binding groove seem to affect activity, some of which are quite far from the active site. Substrate chain length also seems to be important for activity, which is probably due to effects on processivity. Even without changing the active site, different product patterns and reaction energies emerge.

Molecular modeling is also part of the project, and the initial results from the experimental studies will give input to simulations that again can give further ideas about what to explore in the laboratory. These studies holds the potential to unravel how substrate binding, epimerization and processivity of epimerases work together for the design of tailor-made alginate.

Structure and function of novel CAZymes

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Keywords: CAZyme discovery, functional and structural characterization

Glycans are among the most variable biological structures in nature. Oligo- or polysaccharides are involved in a variety of biological functions ranging from structure (plant wall), reserve (glycogen), to complex intra and extracellular signaling. Unlike proteins that are encoded by DNA, the structure of glycans is not based on a specific code but is dictated only by the biosynthetic enzymes.

Since 1998, the CAZy database provide a robust sequence-based classification of enzymes involved in glycan degradation, synthesis and modification (www.cazy.org [1]). During the last 15 years, genomic and metagenomic sequencing has increased drastically the number of CAZyme sequences creating an ever-growing gap with the number of CAZymes that have been functionally characterized. Reliable methods for the functional prediction of genomic sequences are thus desirable. Bioinformatics and wet biochemistry form a virtuous partnership where the former can help choose CAZymes to study and the latter ultimately improve functional assignments in genomes and metagenomes.

The combination of PUL prediction and high throughput protein production is a powerful method to discover novel CAZymes and novel CAZyme families. In this poster we will present examples of the functional and structural results that we have obtained recently using this approach.

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Structure-function relationships underlying the dual specificity of the peptidoglycan deacetylase *Bs*PdaC

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Keywords: *Bs*PdaC, CE4 family, chitooligosaccharides, 3D structure.

Peptidoglycan GlcNAc deacetylases and MurNAc deacetylases are classified in carbohydrate esterase family 4 (CE4) from CAZy database together with chitin deacetylases, poly-ß1,6-*N*-acetylglucosamine deacetylases and acetylxylan esterases. *Bacillus subtilis* PdaC (*Bs*PdaC) is a peptidoglycan *N*-deacetylase acting on *N*-acetylmuramic acid (MurNAc) that also shows *N*-acetylglucosamine deacetylase activity on chitooligosaccharides [1].

Chitosans and chitooligosaccharides are bioactive molecules with many current and potentially new applications in several fields and their physicochemical and biological properties are determined by their specific structures. Since chemical deacetylation methods yield products with a random pattern of acetylation, there is a growing interest in developing selective enzymatic approaches to produce sequence-defined COS to evaluate their biological functions and develop new applications.

In this context, CE4 enzymes active on chitin oligomers show great potential for their use as biocatalysts. The deacetylation patterns exhibited by these enzymes are diverse, some being specific for some positions of the oligomeric substrates and others following a multiple attack or processive mechanisms leading to partially or fully deacetylated products. To rationalize these differences in the pattern exhibited by different CE4 family members and, based on the first 3D-structure of a CDA in complex with substrates, we proposed the "Subsite Capping Model" [2,3]

To better understand the determinants of substrate specificity and also the molecular bases of the dual activity showed by *Bs*PdaC, we determined the X-ray 3D structure of the CE4 catalytic domain of the enzyme at a 1.54Å resolution and analyzed the mode of action on chitooligosaccharide substrates [4]. The enzyme deacetylates COS with DP ≥3 following a multiple attack mechanism in which all but the reducing-end GlcNAc residues are deacetylated. Docking of substrates revealed the protein-ligand interactions that define the initial deacetylation events experimentally observed, which are guiding engineering strategies to modify the substrate specificity.

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Structural determinants of GH13 α-glucan debranching activity and its natural endogenous regulation

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Keywords: α-glucan debranching enzyme, starch degradation, limit dextrinase, limit dextrinase inhibitor

α-Glucan debranching enzymes (DBEs) hydrolyse α-1,6-linkages in starch and glycogen, playing a central role in energy metabolism in all living organisms and also include very important industrial enzymes. Although crystal structures are available of several DBEs [1], only that of barley limit dextrinase (LD) is determined in complex with a branched natural substrate as well as with a range of maltooligosaccharide products [2]. Moreover, the structure is available of LD in complex with the endogenous limit dextrinase inhibitor (LDI) that binds to LD with picomolar affinity [3]. Altogether the enzymatic reactions and regulation of LD are known with exceptional structural detail. LD and other pullulanase-type DBEs have N-terminal modules of unknown function and not seen to bind substrate [1]. In cereal LDs this domain is a distant structural homologue of family CBM21 [4]. Remarkably, a minor allele sorghum line has increased debranching activity. The minor allele encodes LD with two point-mutations in the N-terminal domain [5]. Corresponding single and double barley LD mutants situated about 45 Å from the active site, were produced in *Pichia pastoris* [6] and showed up to 85% loss in catalytic efficiency towards amylopectin, β-limit dextrin and pullulan indicating a central role of the N-terminal domain in activity. Compared to other DBEs cereal LDs have high preference for α-limit dextrins and pullulan over amylopectin. The surprising equally critical effect on activity for oligo- and polysaccharides by engineering subsite +3 of the substrate binding cleft provides novel insight into structural determinants of α-glucan specificity. Finally, computational guided mutational analysis of the natural LDI control of barley LD indicated that the picomolar binding affinity depends on both an intermolecular hydrophobic cluster formed at the LDI/LD interface and several residues at the rim of the LDI/LD complex. This is in contrast to the idea of having a central hot-spot, which drives the affinity.

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Probing the functional diversity within fungal auxiliary activity family 7 (AA7) oxidases

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Keywords: oxidoreductases, auxiliary activity, chitooligosaccharide, *Aspergillus nidulans*

Interactions between fungi and plants are of tremendous socio-economic impact in terms of pathogenesis, biomass recycling, production of value-added chemicals and biofuels. Fungi secrete a multitude of enzymes of different classes to degrade naturally abundant biopolymers such as lignocellulose, chitin and starch [1]. The oxidoreductase fraction of fungal secretomes is a rich source of biocatalysts that possess significant potential for utilization in a variety of biotechnological processes, e.g. as biofuels, bio-oxidants in biofuel cells and biosensors.
Previously, we have described the co-secretion of a multitude of LPMOs and other AA oxidoreductases together with hydrolytic enzymes during the growth of *Aspergillus nidulans* [2]. Proteomic analysis of these fungal secretomes revealed the co-secretion of several enzymes that belong to the AA7 family. These putative glyco-oligosaccharide oxidases display FAD-binding and Berberine bridge enzyme (BBE)-like domains. Some members of the AA7 family have been shown to accommodate oligosaccharides including cello-, xylo, chito- and malto-oligosaccharides [3, 4]. All these substrates are oxidized at the C1 position to the corresponding lactones. To date, the functional diversity and the substrate recognition within the AA7 family are not well-understood.
We determined the crystal structure of a chitooligosaccharide AA7 oxidase from *Fusarium graminearum* at 1.8 Å resolution and characterized this enzyme biochemically. This first structure of a chito-oligosaccharide active AA7 candidate is shown to exploit a novel active site architecture for recognition and oxidation of oligosaccharides with an *N*-acetyl moiety at C2 of the reducing end.
In addition, these data promote our understanding of the structural elements that contribute to the remarkable functional diversity and specificity within this family of enzymes.

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Starch-binding domains as CBM families – history, occurrence, structure, function and evolution

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Keywords: starch-binding domains, CBM families, structural comparison, evolutionary relationships.

The term "starch-binding domain" (SBD) has been applied to a domain within an amylolytic enzyme that gave the enzyme the ability to bind onto raw, i.e. thermally non-treated, granular starch [1]. An SBD is a special case of a carbohydrate-binding domain (CBM), which in general, is a structurally and functionally independent protein module exhibiting no enzymatic activity but possessing potential to target the catalytic domain to the carbohydrate substrate to accommodate it and process it at the active site [2]. As so-called families, SBDs together with other carbohydrate-binding modules have become an integral part of the CAZy database (http://www.cazy.org/). The first two SBDs, i.e. the C-terminal *Aspergillus*-type and the N-terminal *Rhizopus*-type have been assigned the families CBM20 and CBM21, respectively [2,3]. Currently, among the 84 established CBM families in CAZy, fifteen can be considered as families having SBD functional characteristics: CBM20, 21, 25, 26, 34, 41, 45, 48, 53, 58, 68, 69, 74, 82 and 83 [3]. All known SBDs, with the exception of the extra long CBM74 [4], were recognized as a module consisting of approximately 100 residues, adopting a β-sandwich fold and possessing at least one carbohydrate-binding site [5-7]. The main attention will be paid to presenting: (i) the SBD identification in different amylolytic and related enzymes (e.g., GH families) as well as in other relevant enzymes and proteins (e.g., laforin, the β-subunit of AMPK, and others); (ii) the information on the position in the polypeptide chain and the number of SBD copies and their CBM family affiliation (if appropriate); (iii) the structure/function studies of SBDs with a special focus on solved tertiary structures, in particular, as complexes with α-glucan ligands; and (iv) the evolutionary relationships of SBDs in a tree common to all SBD CBM families (except for the extra long CBM74). Finally, some special cases and novel potential SBDs will also be introduced.

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Five function-unknown GH97 paralogs from *Bacteroides thetaiotaomicron*

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Keywords: GH97, *Bacteroides thetaiotaomicron*, molecular evolution

Glycoside hydrolase family 97 (GH97) is a distinctive family, in which the enzymes diverge into inverting and retaining glycosidases. *Bacteroides thetaiotaomicron* has ten GH97 paralogs and five of them have been characterized: inverting α-glucoside hydrolase (BT3703), retaining α-galactosidases (BT1871, BT2620, BT3664) and β-L-arabinopyranosidase/α-galactosidase (BT3661). In this study, we characterize other paralogs, BT0132, BT0683, BT3163, BT3294 and BT4581, to obtain insights into molecular evolution of GH97 glycosidases.

Recombinant BT0132, BT0683, BT3163, BT3294 and BT4581 were produced using *Escherichia coli*, and then purified by Ni^{2+}-affinity column chromatography. To evaluate their substrate specificity, the hydrolysis activities of recombinant enzymes toward *p*-nitrophenyl (*p*NP) glycosides were examined. We identified BT0683 and BT4581 as α-glucoside hydrolase, since they considerably hydrolyzed *p*NP α-D-glucopyranoside (*p*NP Glc): their k_{cat}/K_m values for *p*NP Glc were 370 $s^{-1}mM^{-1}$ and 5000 $s^{-1}mM^{-1}$, respectively. BT3163 and BT3294 exhibited appreciable hydrolysis activity toward *p*NP α-D-galactopyranoside (*p*NP Gal) with k_{cat}/K_m values of 0.24 $s^{-1}mM^{-1}$ and 8.9 $s^{-1}mM^{-1}$ for *p*NP Gal, respectively, so that we ascertained that both enzymes were α-galactosidases. BT0132 hydrolyzed *p*NP Glc; however, the hydrolytic rate was so low as not to be able to deem BT0132 to be α-glucoside hydrolase.

We further analyzed the substrate specificity for oligosaccharides. At first, the kinetic parameters of BT0683 and BT4581 for glucobioses were determined. BT0683 showed a high specificity for nigerose: the k_{cat}/K_m values for maltose, isomaltose, kojibiose and nigerose were 0.011, 0.036, 13 and 120 $s^{-1}mM^{-1}$, respectively. BT4581 exhibited a wide specificity toward glucobioses: the k_{cat}/K_m values of BT4581 for maltose, isomaltose, kojibiose and nigerose were 30, 130, 160 and 100 $s^{-1}mM^{-1}$, respectively. BT4581 displayed higher specificity for isomaltose than BT3703, of which substrate specificity had been well characterized [1].

Kinetic parameters of BT3163 and BT3294 for melibiose and raffinose were determined. BT3163 hydrolyzed melibiose with the k_{cat}/K_m values of 3.1×10^{-4} $s^{-1}mM^{-1}$, but did not raffinose. BT3294 hydrolyzed melibiose and raffinose at the similar rate (k_{cat}/K_m values: 0.66 and 1.0 $s^{-1}mM^{-1}$, respectively). This specificity is diverged from BT1871, which exhibited higher specificity for melibiose than raffinose.

This study demonstrates that substrate recognition mechanism evolved in each subfamily after the catalytic mechanism had forked.

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The influence of N-glycosylation on the activity of cellobiohydrolyase Cel7A from *Trichoderma reesei*

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Keywords: cellulase, cellobiohydrolase, glycosylation, enzyme kinetics

Glycosylation is a commonly observed post-translational modification of fungal cellulases. The typical architecture of cellulases includes N-glycosylated catalytic domain, which is connected to carbohydrate-binding module through a flexible, O-glycosylated linker peptide. Functional roles of the N-glycosylation have been widely studied, but remain elusive. Most attempts to elucidate effects of glycosylation on cellulase activity have used end-point measurements or conventional Michaelis-Menten theory, which may not be fully descriptive if the cellulase activity is tested on insoluble substrate.

In the current work, we have studied effects of glycans on cellulase activity using a broader kinetic approach. Specifically, we investigated the wild type and an N-glycan knockout of the cellobiohydrolase Cel7A from *T. reesei*. Our kinetic analysis included both substrate saturation ($E_0 << K_M + S_0$; conventional MM) and enzyme saturation ($S_0 << K_M + E_0$, inverse MM), and the results revealed an interesting higher number of attack sites on the cellulose surface and changed binding capacity for the N-glycan mutant.

Probing the dynamics of a starch-active AA13 LPMO

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Keywords: AA13, Lytic polysaccharide monooxygenases, Molecular dynamics simulations, Starch.

Lytic polysaccharide monooxygenases (LPMOs) are copper-dependent monooxygenases, which utilize hydrogen peroxide or molecular oxygen and extrinsic electron sources to oxidize glycosidic bonds of polysaccharides at the C1 or C4 position. LPMOs from auxiliary activity family AA13 in CAZy have been shown to be active on starch [1]. Starch-active LPMOs possess a shallow binding site to accommodate the helically organized amylose or amylopectin polymers of starch in contrast to cellulose-active counterparts with a flatter architecture [2]. In order to access the supramolecular insoluble starch, LPMOs of AA13 frequently occur as modular enzymes joint to a starch binding module from the carbohydrate binding module family 20 (CBM20) [3]. Proteomic analysis of fungal cultures growing on starch emphasized the abundance of both the isolated LPMO13 and the modular LPMO13-CBM20 in the starch secretomes [4]. Notably, the details of the mode of action of LPMO13 and their interaction with starch remain unknown.

We performed molecular dynamics simulations and docking studies on LPMO13 based on the only structurally characterized LPMO to explore the dynamics and substrate recognition by this enzyme to further progress the structural understanding of these starch-active enzymes.

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Structure, activity, dynamics and stability of a multimodular xanthan lyase from *Paenibacillus nanensis*

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Keywords: provide four keywords separated by commas.

Paenibacillus nanensis Xanthan lyase (PXL) is a carbohydrate modifying enzyme involved in the degradation of xanthan and consists of a PL8 catalytic domain (as classified in CAZy, http://www.cazy.org) followed by two additional C-terminal modules. The structure of the full length 113 kDa enzyme was determined by X-ray crystallography [1], representing the second xanthan lyase catalytic module to be structurally characterized in PL8 (the other being *Bacillus* sp. GL1 Xanthan Lyase (BXL) [2]. Unlike other xanthan lyases including BXL, PXL is specific for both unmodified mannose and pyruvylated mannose, correlating with structural features in the substrate binding groove [1]. The two additional C-terminal modules share a similar β-sandwich architecture also common to CBM6/CBM35/CBM36. The first of these modules belongs to the newly defined CBM family 84, found also associated to hydrolases involved in xanthan degradation [3]. The second is not yet classified as a CBM in CAZy, due to lack of biochemical evidence for carbohydrate binding, but is likely to represent an additional CBM family. Solution analysis by Small Angle X-ray Scattering (SAXS) reavealed a relatively extended conformation with some degree of flexibility [1].

The PXL activity and stability was highly dependent on the presence of Ca^{2+}. The conformational impact of Ca^{2+} (and its removal) on PXL was investigated by hydrogen/deuterium exchange mass spectrometry (HDX-MS). Together with the crystal structure, this information guided the rational engineering of PXL variants that showed improved stability and activity under calcium chelating conditions, which are favourable for new industrial applications of PXL [1].

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The NMR structure of carbohydrate binding module 14 from human chitotriosidase and its interaction with chitin

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Keywords: Carbohydrate binding module, CBM14, chitin, NMR

Protein-carbohydrate interactions are involved in numerous biological processes (e.g. antigen-antibody recognition, enzyme specificity, cell-cell adhesion) and understanding them is critical for the advancement of future medicine and biotechnology. A key element for elucidating such interactions is to study the structural changes of the protein as it interacts with a substrate in solution.

We have investigated carbohydrate binding module 14 (CBM14), a non-catalytic module of human chitotriosidase (HCHT) that recognizes and binds chitin. The biological function of HCHT is still unclear, but it is believed to be involved in innate immunity. Here we have used NMR to solve the solution structure of CBM14 and probe its interaction with substrate. Furthermore, the interaction between the substrate and a CBM14 Leu454Ala mutant has been characterized. This has revealed that the Trp465 involved in binding chitin needs to be oriented correctly and properly exposed to be able to bind efficiently. Mutation of Leu454 causes Trp465 to be buried deeper into the hydrophobic core of the structure, resulting in a detrimental effect on the CBMs ability to bind chitin. Previous investigations of this protein have been done using chito-oligomers as substrate, as this CBM typically binds shorter oligomers. In our studies we have investigated its binding to β-chitin, and revealed that positive amino acids on the protein's surface forms polar interactions with the substrate.

Diversity within GH family 3 - Characterization of six enzymes from *Rhodothermus marinus*

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Keywords: GH3, β-glucosidase, β-xylosidase, β-*N*-acetylglucosaminidase.

Glycoside hydrolase family 3 (GH3) encompass a wide array of activities, and its members are interesting due their potential role in biomass degradation, cell wall remodeling and recycling, and in pathogen defense [1]. GH3 members are exo-acting, retaining enzymes with activities such as β-glucosidase, β-xylosidase, β-*N*-acetylglucosaminidase and α-L-arabinofuranosidase. Glycoside hydrolases are assigned to their respective GH family based on amino acid sequence similarity, however, it is difficult to resolve their substrate specificity without biochemical investigation.

The genome of the thermophilic marine bacterium *Rhodothermus marinus* DSM 4253 encodes six enzymes classified under GH3. The six enzymes have non-redundant substrate specificities and has been biochemically, phylogenetically and structurally studied. The aim was to identify features important for substrate specificity within GH3 based on amino acid sequence, rather than labor intense biochemical characterization.

The substrate specificities of the six enzymes from *R. marinus* could be related to sequence-based subgrouping within GH3, structural features, conserved motifs and domain composition. *Rm*Nag3 represents the deepest root within the phylogenetic tree of GH3 which consists of β-*N*-acetylglucosaminidases and which can be identified by a conserved motif including the Asp-His dyad in domain 1 [2]. The three β-glucosidases, *Rm*Bgl3A-C, represent different evolutionary lineages and showed different specificities. For *Rm*Bgl3A, a relative long linker region between domain 2 and a FnIII domain was involved in subsite +1. Two interacting residues on the linker were also found in closely related enzymes. *Rm*Bgl3B showed activity only on short cellooligosaccharides, longer substrates were sterically hindered by a PA14 domain. *Rm*Bgl3C showed a preference for β-1,3-linkages and displayed a tryptophan on loop j in domain 2. This residue has been found in other GH3 enzymes which cleave β-1,3-linkages. The two closely related β-xylosidases, *Rm*Bgl3A and B, clustered within one out of two evolutionary lineages of β-xylosidases. Both enzymes displayed a glutamic acid on β-strand c in subsite -1, instead of an aspartic acid as found in the β-glucosidases. These residues were found to be conserved between β-xylosidases and β-glucosidases within the entire GH3. These findings present significant insights into GH3 which will facilitate future characterization of enzymes within the family.

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Chemoenzymatic synthesis of novel substrates for the facile measurement of glucuronyl esterase and α-glucuronidase

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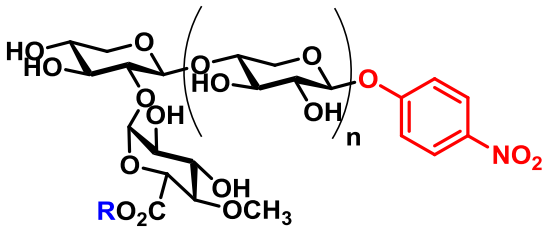
Keywords: enzyme coupled assays, colourimetric substrates, automated assays.

Glucuronyl esterases (GE) constitute a recently discovered class of enzymes, first reported in 2006 [1]. These enzymes are involved in delignification, hydrolysing the ester linkages between lignin polyphenol structures and the glucuronoxylan present in the hemicellulose fraction of lignocellulosic biomass. Given that these linkages are believed to contribute to the recalcitrant nature of lignocellulose, the discovery of new and improved glucuronyl esterases is an important target for the biofuel industry. A number of synthetic substrates to measure GE activity have previously been described in the literature [2]. Building on these seminal studies, we prepared a range of substrates (**1**-**4**) which, when combined with two ancillary enzymes, namely a GH67 α-glucuronidase from *Geobacillus stearothermophilus* and a GH43 β-xylosidase from *Selenomonas ruminantium*, form the basis of a colourimetric assay for glucuronyl esterases.

α-Glucuronidases cleave the uronic acids decorating the xylan backbone in hemicellulose [3]. While a commercial enzymatic assay kit for the measurement of α-glucuronidase activity based on uronate dehydrogenase (UDH) mediated detection has been available for a number of years, we sought to develop a new colourimetric assay that would further simplify the measurement of this enzyme. Substrate **5** was combined with the β-xylosidase described above to form the basis for the colorimetric assay of α-glucuronidase.

The structural requirements for a sensitive substrate and the chemoenzymatic preparation of these compounds will be discussed along with the development of the required enzyme coupled assay formats and their application to the measurement of glucuronyl esterase and α-glucuronidase.



1: n=2; R=CH_3
2: n=2; R=Bn
3: n=2; R=$(CH_2)_3Ph$
4: n=1; R=CH_3
5: n=1; R=H

Figure 1. Substrates for the measurement of glucuronyl esterase (**1**-**4**) and α-glucuronidase (**5**).

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The N-terminal module of a bacterial group of family GH77 amylomaltases – a possible new starch-binding domain

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Keywords: amylomaltase, N-terminal domain, starch-binding domain, tertiary structure comparison.

Within the CAZy database [1], 4-α-glucanotransferases (EC 2.4.1.25) have been classified also in the family GH77 of glycoside hydrolases. The GH77 is a monospecific family [2] and forms the clan GH-H with the main α-amylase family GH13 and the family GH70 [3]. The enzyme 4-α-glucanotransferase is known also as amylomaltase in prokaryotes [4,5] or disproportionating enzyme in plants [6]. It catalyses the transfer of an α-glucan chain from one α-1,4-glucan to another α-1,4-glucan, or the production of a cyclic α-1,4-glucan within a single linear glucan molecule [4-6]. Amylolytic enzymes, which are capable of binding and degrading raw starch, usually possess a distinct sequence-structural module, the so-called starch-binding domain (SBD) [7]. These domains help to increase the binding and degradation of raw starch by amylolytic enzymes. Within the family GH77, a group of bacterial amylomaltases with the N-terminal extension, represented by those from *Escherichia coli* and *Corynebacterium glutamicum*, was identified [2]. A substantial part of the extension forms a distinct domain, for which the exact function has not been determined yet [8,9]. A detailed bioinformatics analysis of more than 450 sequences of homologues of this N-terminal domain from family GH77 revealed several residues, e.g., G107, Y108 and P128 (MalQ from *E. coli* numbering), as well conserved positions. Since the comparison of N-terminal modules of both GH77 amylomaltases (*E. coli* and *C. glutamicum*) with already established real SBD CBM families did not reveal any significant structural similarity, the N-terminal domain could represent a novel, until now non-characterized CBM. This hypothesis can be supported by results of docking studies trying to identify the eventual binding residues for linear maltooligosaccharides (M2, M3, M4) and β-cyclodextrin. Among them, the Y108 from *E. coli* amylomaltase and its counterpart W143 from *C. glutamicum* enzyme seem to be the best candidates for the α-glucan binding. Currently, based on the *in silico* study only, it is not possible to decide unambiguously whether or not the N-terminal module of this bacterial group of GH77 amylomaltases, represented by the enzymes from *E. coli* and *C. glutamicum*, may define a novel CBM family.

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Exploration of the molecular determinants involved in alternansucrase specificity and polymerization

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Keywords: GH70, glucansucrase, alternansucrase, crystal structure, specificity

The alternansucrase (ASR) from *Leuconostoc citreum* NRRL B-1355 is an α-transglucosylase belonging to the family 70 of glycoside hydrolases (GH70). The glucansucrases from this family use a cheap and abundant substrate, sucrose, to catalyze the formation of high molar mass (HMM) homopolymers of glucosyl units, named α-glucans, displaying interesting properties for food, feed or health applications. ASR stands apart among these glucansucrases, being the only one to produce an intriguing α-glucan, the alternan, made of alternating α-1,6 and α-1,3 linkages in the main chain [1]. ASR produces a bimodal population of both HMM and lower molar mass (LMM) alternan [2]. Additionally, ASR has a 45°C optimum temperature [3], being one of the most stable glucansucrases known to date. However, in the absence of 3D structural data, most of the ASR determinants involved in linkage specificity, polymerization or higher stability remain unraveled. To better understand ASR molecular determinants, we have solved the 3D structure of this enzyme both unliganded and in complex with various sugar ligands. Combined to mutant biochemical characterization, our results enable the identification of residues defining the +2 and +2' subsites, in the prolongation of ASR subsites -1 and +1. The positioning of acceptor in either +2 or +2' subsite is proposed to control the linkage specificity in α-1,6 or in α-1,3 linkage formation respectively. The analysis of the sugar ligand complexes shed the light on another site, more distant but also located in domain A, that was shown to be involved in HMM alternan formation and anchoring. This site is a signature of ASR and could act as a bridge facilitating alternan processive elongation. Bringing all together, our results have deepened the knowledge on ASR structure, specificity and has allowed structural determinants involved in polymerization to be unraveled. Overall, they open new paths of investigation for structure-function relationship studies of glucansucrases and for the conception of polymers with controlled structures and physico-chemical properties.

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Insight into aglycone specificity of GH130_2 mannoside phosphorylase

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Keywords: GH130, mannoside phosphorylase, mannooligosaccharide.

Glycoside hydrolase family 130 is composed of β-mannoside-active phosphorylases and hydrolases, which fall into two subfamilies and a non-classified group. Subfamily 1 enzymes are 4-*O*-D-mannosyl-D-glucose phosphorylase (MGP, EC2.4.1.281), which phosphorolyzes Manβ1-4Glc to βMan1*P* and Glc. MGP is possibly involved the mannan metabolism in bacteria through the CE-MGP pathway, in which (β1-4)-mannobiose (Man_2) is first epimerized by cellobiose 2-epimerase and then the produced Manβ1-4Glc is phosphorolyzed by MGP. Subfamily 2 (GH130_2) is composed of more diverse activities, namely β-1,4-mannooligosaccharide phosphorylase (MOP, EC2.4.1.319), 1,4-β-mannosyl-*N*-acetylglucosamine phosphorylase (EC2.4.1.320), and β-1,4-mannosylchitobiose phosphorylase. All of them act on β-mannosidic linkages, but their aglycone preference of substrates is diverse.

In this study, one of the GH130_2 enzyme, TmMOP (TM1225) from an thermophilic anaerobe *Thermotoga maritima*, was kinetically and structurally analyzed to provide insight into molecular mechanism of the substrate recognition. The highest preference, on the basis of apparent k_{cat}/K_m, was observed on Man_5 among Man_2-Man_6 in phosphorolysis. In the reverse phosphorolysis in the presence of 10 mM Man1*P*, D-Man was the best acceptor substrate, while no activity on GlcNAc and weak activity on Glc were detected. Crystal structure of TmMOP, determined in a complex with Man_3, suggested that a Trp residue in a loop connecting blade-3 and blade-4 in the five-bladed β-propeller fold was located close to C2 and C6 of Man residue in subsite +1 and +2, respectively. Its mutant enzyme W207A drastically lost its reverse phosphorolytic activities towards Man and Man_2, but kept activity on Glc and acquired activity on GlcNAc. The Trp residue is not conserved in GH130_2 Man-GlcNAc phosphorylases. Therefore, the Trp residue is regarded as a key residue in GH130_2 enzymes, essential for mannooligosaccharide binding and excluding Man-GlcNAc from substrate.

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Structural and functional characterization of an unclassified enzyme with ability to deacetylate and breakdown xylans

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Keywords: carbohydrate esterase, crystal structure, α/β-hydrolase

Proteins of unknown function were identified in polysaccharide utilization loci (PUL) encoded by a beaver gut microbiome enriched on pretreated hardwood; corresponding proteins were recombinantly expressed in *E. coli* for functional characterization. One of the selected proteins, herein referred to as "Unk1", was encoded by a PUL comprising carbohydrate-active enzymes predicted to act on complex carbohydrates, including xylans. Unk1 was shown to catalyze the deacetylation of xylo-oligosaccharides. Moreover, Unk1 displayed glycoside hydrolase activity towards glucuronoxylan and arabinoxylan. To investigate the structural basis for the apparent dual esterase/glycoside hydrolase activity of Unk1, the crystal structure of the enzyme was solved at 1.85Å resolution through phenix.mr_rosetta [1] software that used 250 model structures to find molecular replacement solution.

The overall structure is a typical α/β-hydrolase fold, where eight mainly parallel beta-strands are flanked by alpha-helixes. As observed for other carbohydrate esterases, the active site is formed by a catalytic triad (Ser128, Asp237, His269) located on the surface of the protein within a surface exposed beta-sheet. Unk1 was distinguished by an additional, small beta-sheet also on the surface of the protein, not observed in any other carbohydrate esterases. The N-terminus protruded from the protein and formed a small beta-strand that packed against the corresponding beta-sheet of a neighboring molecule. The unique swapped domain stacking of N-terminus causes an open-ended oligomer-type of packing in the crystal. The role of N-terminus in the protein packing was investigated by constructing a truncated form of Unk1. Truncation, however, did not affect the enzyme activity or the packing in the crystal.

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Structural insights in starch conversion by GtfB glucanotransferase enzymes from Lactobacilli

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Keywords: starch conversion, glucanotransferases, digestibility, dietary fiber.

GtfB-type glucanotransferases (GT) from GH70 act on starch and starch-derived oligosaccharides, synthesizing α-glucans. The *Lactobacillus reuteri* 121 4,6-α-GT synthesizes linear isomalto-maltooligosaccharides (IMMP) with up to 95% α-1,6 linked glucosyl units at the non-reducing end; its structure [1] features a tunnel in agreement with its preference for amylose over amylopectin as substrate. Recently, GtfB-type enzymes were identified in the Nestlé Culture Collection (NCC) that convert **both** amylose and amylopectin, synthesizing branched α-glucans with either α-1,4 / α-1,6 linkages [2] or α-1,4 / α-1,3 linkages [3].

In this study we determined the crystal structure of the 4,6-α-GT from *L. reuteri* NCC 2613 (Fig. 1); a more open binding groove agrees with its broader starch-substrate specificity. A complex with acarbose pinpoints acceptor subsites, allowing us to map the full binding groove and identify residues that contribute to acceptor (and product) specificity.

Finally, a homology model of the 4,3-α-GT from *L. fermentum* NCC 2970 was constructed (Fig. 1). Together, the three structures/models of different GtfBs allow us to better understand the different reaction specificities observed in these enzymes. Such insights are important regarding biosynthetic conversion of starch to α-glucans with reduced digestibility (dietary fiber-like and/or prebiotic properties)[4].

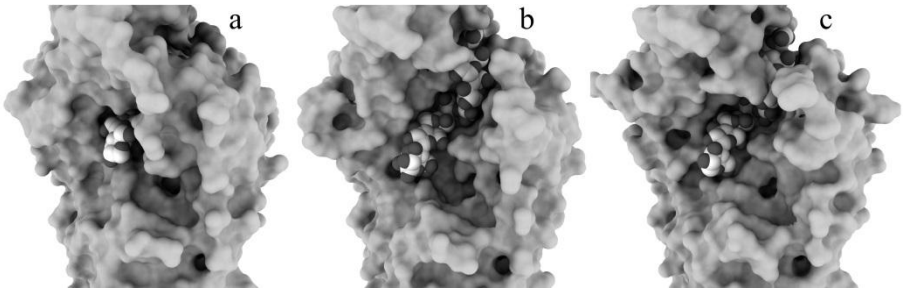

Figure 1. Comparison of GtfB structures: (a) *L. reuteri* 121 4,6-α-GT, (b) *L. reuteri* NCC 2613 4,6-α-GT, (c) *L. fermentum* NCC 2970 4,3-α-GT with modeled maltooctaose (spheres).

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Systematic deletions in *Trichoderma reesei* Cel7A reveal functional roles of the flexible loops

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Keywords: cellulases, GH7, protein engineering, enzyme kinetics.

Family 7 cellulases (GH7) are some of the most efficient enzymes for cellulose degradation. This makes them particularly relevant for industries producing sustainable fuels from lignocellulosic biomass feedstocks. The secretome of the cellulolytic model fungus *Trichoderma reesei* contains two GH7s, termed *Tr*Cel7A and *Tr*Cel7B. Despite high structure and sequence homology, these enzymes are very different with respect to function. *Tr*Cel7A is an exolytic, processive cellobiohydrolase (CBH), with high activity on crystalline cellulose, while *Tr*Cel7B is an endoglucanase (EG) with specificity for more amorphous cellulose. On a structural level, these functional differences are usually ascribed to the flexible loops that cover the substrate-binding area: *Tr*Cel7A has an extensive tunnel created by eight peripheral loops, while the absence of four of these loops in *Tr*Cel7B makes its catalytic domain a more open cleft [1]. To investigate structure-function relationships of such loops, we produced and kinetically characterized a family of variants in which four unique loops in *Tr*Cel7A were individually deleted, in order to resemble the structure of *TrCel7B* [2]. Analysis of a range of kinetic parameters consistently showed that the B2 loop, covering the substrate-binding subsites -3 and -4 in *Tr*Cel7A, was a key determinant for the distinction between CBH and EG-like behavior of *Tr*Cel7A and *Tr*Cel7B. We surmise that these results could be useful both in mechanistic discussions and as guidance for engineering this industrially significant group of enzymes.

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Functional global flexibility demonstrated for a multi-domain GH43 arabinanase

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Keywords: glycoside hydrolase, GH43, arabinanase, conformational dynamics

*Gs*Abn43A is an extracellular α-L-arabinanase from the thermophilic bacterium *Geobacillus stearothermophilus*-T6 [1]. The 3D structure of *Gs*Abn43A has recently been determined by X-ray crystallography, representing the largest structure reported so far in the GH43 family, and revealing a unique "pincer-shaped" architecture composed of four domains (**Figure 1a**). Homolog structures of two of these domains (D1,D2) have been reported [2,3], but the other two domains (D3,D4) are unique to *Gs*Abn43A. One of these domains (D4) represents a new carbohydrate-binding module (CBM) family, to which an arabinopentaose substrate was found bound (**Figure 1a**). Three different crystallographic conformational states have been determined for *Gs*Abn43A, differentiated by approximately 13 Å movement in location of D3 and D4. Complementary analysis conducted by small angle X-ray scattering (SAXS), dynamic light scattering (DLS), molecular dynamics (MD) and metadynamics, all suggest that these conformation changes are significantly larger in solution, involving global movements of up to 100 Å (**Figure 1b**). Mutagenesis and kinetic experiments indicate that such global flexibility is also functional, suggesting a "harpoon" catalytic mechanism for arabinan degradation by *Gs*Abn43A.

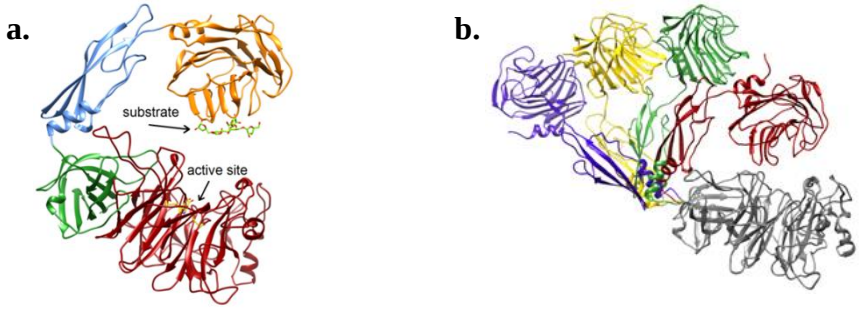

Figure 1: **a.** The crystal structure of a *Gs*Abn43A conformer, displaying its four domains.
b. The large global conformational changes demonstrated for *Gs*Abn43A.
(The grey domains (D1,D2) are fixed, the colored domains (D3,D4) move)

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It´s in the heat; the mode of glycoside hydrolase interaction with nature's largest reservoirs of organic carbon revealed.

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Keywords: provide four keywords separated by commas.

Chitin and cellulose represent some of nature's largest reservoirs of organic carbon in the form of monomeric hexose sugars (*N*-acetyl-glucosamine and glucose, respectively) linearly linked by β-1,4 glycosidic bonds. In their natural form, both polysaccharides are organized in crystalline arrangements that make up robust biological structures including crustacean cuticles (chitin) and plant cell walls (cellulose). Although this crystalline construct is crucial for biological function, it presents a significant challenge in industrial utilization of biomass, where efficient enzymatic depolymerization is a critical step. Enzymatic degradation of recalcitrant polysaccharides is thought to occur through the synergistic action of glycoside hydrolases (GHs) that have complementary activities. Endo-acting GHs make random scissions on the polysaccharide chains, whereas exo-acting GHs mainly target single reducing and non-reducing chain ends.

Processive GHs, both endo- and exo-acting, closely associate with polymer chains and repeatedly cleave glycosidic linkages without dissociating from the crystalline surface and are typically the most abundant enzymes in both natural secretomes and industrial cocktails by virtue of their significant hydrolytic potential. We have over the last 12 years investigated the interactions of GHs active on chitin and cellulose with respect to correlation of thermodynamic signatures of substrate binding with mode of action using isothermal titration calorimetry (ITC). Thermodynamics signatures of binding are a function of the geometry, dynamics, and chemical composition of the substrate tunnels or clefts. Here, we see that there is a correlation between binding free energy and enthalpy change with processive ability, enthalpy change with reducing end specificity, and changes in solvation entropy with active site topology. The talk will mainly focus on lessons learned from studying on how a set of co-evolved GHs from a single organism approaches the degradation of a recalcitrant polysaccharide substrate such as cellulose and chitin.

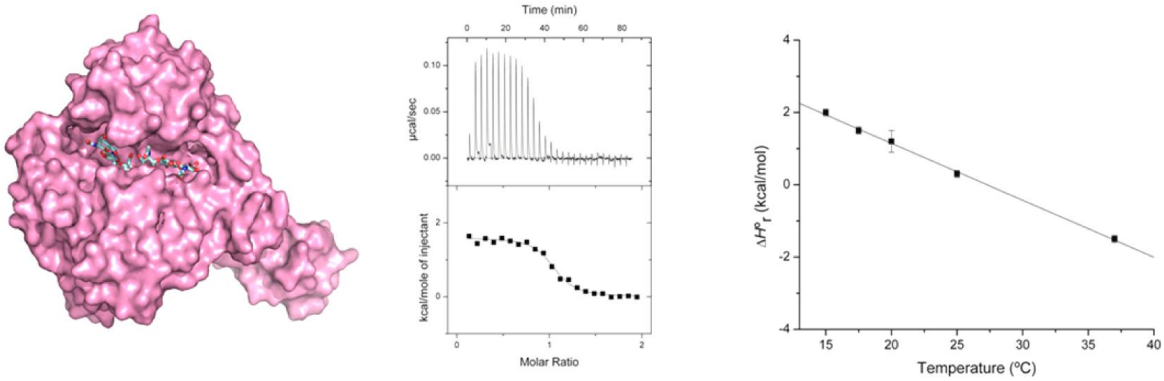

Figure 1. An example of the binding of a substrate to a GH and its study by ITC..

Glycosylate and move: Study of a glycosyltransferase involved in bacterial flagella formation

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Keywords: glycosyltransferase, flagellin, magnetotactic bacteria, nonulosonic acid

Bacterial motility is governed by motor rotation of a filament that is assembled via polymerization of flagellins after secretion via a dedicated Type III secretion system [1]. Many bacteria modify their flagellins through attachment of carbohydrate groups [2], shown to be essential for the formation and functionality of an intact flagellum [3]. The flagellin of our model organism *Magnetospirillum magneticum* AMB-1 (AMB-1) is glycosylated and deletion of the gene maf produced non-glycosylated flagellin and a non-flagellated phenotype. A functional flagellar filament and bacterial motility could be restored by complementation of the deletion mutant. Proteomic analysis revealed that the flagellin of AMB-1 is O-glycosylated with a nonulosonic acid sugar, related to sialic acid. We solved the crystal structure of Maf, which is organised into three domains: a N-terminal Rossman-like domain of unknown function, a central β/α/β domain exhibiting striking structural similarity with sialyltransferases and a C-terminal α-helical bundle with intriguing structural reminiscence of flagellar chaperons. By mutation studies we could identify residues crucial for the glycosyltransferases activity of Maf [4].

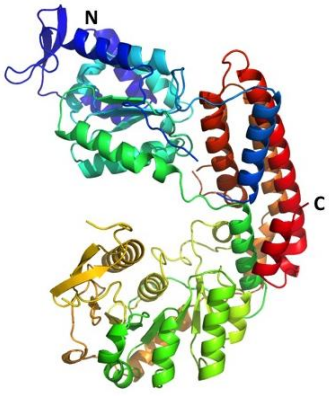

Figure 1. Cartoon representation of the overall structure of Maf.

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Higher order assemblies of GH43 β-xylosidase in solution and in crystal

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Keywords: GH43 β -xylosidase, crystal structure, oligomerization, Mg^{2+} cofactor

We have solved the crystal structure of *Thermobifida fusca* wild type β-1,4-xylosidase, the GH43 enzyme, by molecular replacement at 1.8 Å resolution. The enzyme as being part of an array of hemicellulases is responsible for the complete degradation of plant cell wall polysaccharides. GH43 enzymes are inverting glycosidases, operate through a single displacement mechanism, and are the members of F clan together with GH62 enzymes.

We have found conserved active site topology in the N-terminal catalytic domain of the enzyme, which shows a five-bladed-β-propeller fold. The active site is composed of three catalytic carboxyls surrounded by several conserved aromatic side chains explaining the very strict β-xylosidic bond cleaving ability. The shallow pocket like active site is situated on the opposite face of the very deep water filled tunnel what the five-bladed-β-propeller is created. The C-terminal ß-sandwich domain lost its carbohydrate binding function in evolution and at the same time, it gained an extra anti-parallel β-pair by insertion. This short β-sheet is essential constituent of the active site ensuring the exo-type character of the enzyme.

In crystal, β-xylosidase forms homotetramer, where the C-terminal domain provides a contact area between the two interacting monomers. Two dimers are further assembled into a tetramer with interacting surfaces between loops of the β-propeller in one dimer and loops of the β-sandwich domain in the opposite dimer. Solution studies revealed that the active enzyme is mainly in tetrameric and in octameric oligomerization states. A single Mg^{2+} cofactor binding site is also identified on the C-terminal domain, its functionality will be discussed.

Biochemical characteristics of plant trehalases hydrolyzing trehalose 6-phosphate

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Keywords: trehalase, trehalose, trehalose 6-phosphate, GH37

Trehalose (Tre: α-D-Glc*p*-(1↔1)-α-D-Glc*p*) is a non-reducing disaccharide that is found in major groups of organisms. Generally, Tre is produced through a two-step process: Tre 6-phosphate (Tre6*P*) formation and dephosphorylation of Tre6*P*. Tre, produced, is hydrolyzed to D-glucose by trehalase. In plants, Tre6*P* is an essential signal metabolite, regulating carbon flux and development. Although plants have many isozymes of trehalose-synthesizing enzymes, they possess a single trehalase, belonging to Glycoside Hydrolase family 37 (GH37). GH37 trehalase has an $(\alpha/\alpha)_6$-barrel catalytic domain. The catalytic acid (Asp) and base (Glu) are situated at the α5→α6 and α11→α12 loops, respectively. The α11→α12 loop, which covers the ligand (an inhibitor mimicking Tre) in the complex structure of *Escherichia coli* trehalase (EcTREA), is predicted to move to open the active site upon substrate binding [1]. Trehalases from *Arabidopsis thaliana* and *Oryza sativa* (AtTRE1 and OsTRE1, respectively) have not been enzymatically characterized thus far. In this study, the enzymatic functions of AtTRE1 and OsTRE1 were investigated.

AtTRE1 and OsTRE1, produced in *E. coli* BL21 (DE3) transformant and *Pichia pastoris* X-33 transformant, respectively, were purified by nickel-affinity column chromatography. The specific activities of AtTRE1 and OsTRE1 to10 mM trehalose were 137 U/mg and 184 U/mg, respectively. AtTRE1 showed peaks at pH 3.7 and pH 6.9 in a pH-k_{cat} curve, while it showed a single peak at pH 4.5 in a pH-k_{cat}/K_m curve. These results implied that AtTRE1 forms at least two types of ES complexes (ES_a and ES_n) depending on the reaction pHs. OsTRE1 showed the highest activity pH 4.5, and it kept high activity in a wide pH range. In addition to Tre, both AtTRE1 and OsTRE1 showed hydrolytic activity to Glcα1-α1Xyl, Glcα1-α1Gal, and Glcα1-4-L-Ara, and Tre6*P*. The Tre6*P*-hydrolyzing activity of AtTRE1 at pH 3.7 was significantly higher than at pH 6.9, although AtTRE1 showed a similar k_{cat}/K_m for Tre at pH 3.7 and pH 6.9. For Tre6*P*-binding, open substrate binding site would be required. ES_a of AtTRE1 and the ES complex of OsTRE1 might have open substrate binding site to accommodate Tre6*P*. The Tre6*P*-hydrolyzing activity was not detectable in pig trehalase and have not yet reported in other trehalases. This activity might be specific in plant trehalases.

Sequence comparison of GH37 trehalases showed that amino acid residues interacting 6-OH of D-glucosyl residue in subsite +1 are completely conserved. In plant trehalases, Thr is well conserved at the C-terminal of the α11→α12 loop (EcTREA has Asp at the position). This Thr is far from the binding site. T600D AtTRE1 mutant showed k_{cat}/K_m for Tre comparable to that of a wildtype, but its k_{cat}/K_m for Tre6*P* was half of that of wildtype. Thr600 of AtTRE1 might be involved in the orientation of the α11→α12 loop and alter the hydrolytic activity towards Tre6*P*.

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A fungal *endo*-β-1,2-glucanase with a unique reaction mechanism belongs to a new glycoside hydrolase family

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Keywords: glycoside hydrolase, *endo*-β-1,2-glucanase, fungi, β-1,2-glucan

endo-β-1,2-Glucanase (SGL) is an enzyme that hydrolyzes β-1,2-glucans, which play important physiological roles in some bacteria as a cyclic form [1]. Recently, identification, characterization and structural analysis of bacterial SGLs have been performed, demonstrating a novel member of glycoside hydrolase family (GH144) [2, 3]. However, GH144 does not include any fungal SGL.

We found that a fungal SGL purified from *Talaromyces funiculosus* (*Tf*SGL) has no sequence similarity to any known GHs. The recombinant *Tf*SGL (*Tf*SGLr) specifically hydrolyzed linear and cyclic β-1,2-glucans to sophorose (Glc-β-1,2-Glc). Stereochemical analysis demonstrated that *Tf*SGL is an inverting enzyme. The overall structure of *Tf*SGLr comprises an $(\alpha/\alpha)_6$ toroid fold. The substrate binding mode was determined by obtaining Michaelis complex of an inactive *Tf*SGLr-mutant (E262Q) with a β-1,2-glucoheptasaccharide. Along with this binding mode, mutational analysis and action pattern analysis of β-1,2-glucooligosaccharide derivatives suggest an unprecedented catalytic mechanism as follows. E262 (general acid) indirectly protonates the anomeric oxygen at subsite −1 via the 3-hydroxy group of the Glc moiety at subsite +2 and D446 (general base) activates the nucleophilic water via another water. *Tf*SGLr is apparently different from a GH144 SGL in the reaction and substrate recognition mechanisms based on structural comparison. Overall, we propose that *Tf*SGL and its homologs should be classified into a new family.

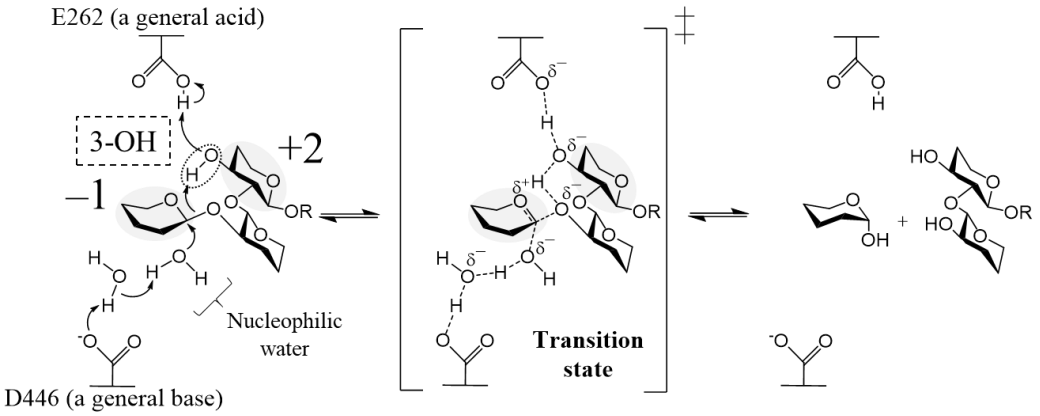

Figure 1. The reaction mechanism of *Tf*SGLr. The Glc moieties at subsites −1 and +2 were highlighted in *gray*.

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Carbohydrate binding module of family 48 enables the action of ferulic acid esterases on polymeric arabinoxylan

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Keywords: CBM48, CE1, ferulic acid esterase, structure-function relationships

Arabinoxylans (AXs) are a major component of hemicelluloses, which is widely distributed in secondary cell walls of plants. Their backbone is composed of β-1,4-linked xylopyranose residues that are single substituted with α-L-1,3-arabinofuranose (Ara*f*) or double substituted with both α-L-1,2- and α-L-1,3-Ara*f*, which can be further substituted with by 5-*O*-ferulic acid and other hydroxycinnamic acids [1]. Ferulic acid esterases (FAEs) catalyze the hydrolysis of ester bonds between hydroxycinnamic acids and Ara*f*, however, most characterized FAEs do not display a preference for polymeric substrates [2,3].

Recently, a number of carbohydrate esterase family 1 (CE1) identified in metagenomic studies was shown to have a carbohydrate binding module of family 48 (CBM48) appended [4,5], a family associated with starch binding [6]. Our phylogenetic analysis demonstrated that these are in fact CBM48s suggesting that CBM48s is a polyspecific family since CE1s do not target starch. Adsorption assays with two CE1-CBM48 enzymes demonstrated binding to AXs, but not to starch, which was supported by a surface plasmon resonance analysis showing no binding to β-cyclodextrin or maltohexaose. Weak binding was detected to arabino- and xylooligosaccharides and interestingly also to maltotetraose. The two CE1-CBM48 enzymes released FA from AXs, while the CE1 domain on its own only released FA from oligosaccharides and unlike the full-length enzymes the CE1 domain was unable to bind to AXs. Crystal structures of the two CE1-CBM48s revealed two integrally folded units and multiple structurally conserved hydrogen bonds fix the CBM48's position relative to the CE1 domains. Docking studies suggest that the xylan main chain is accommodated in the cleft formed at the interface between the CE1 and CBM48 domains.

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How a promiscuous enzyme became selective. The story of genetic engineering.

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Keywords: β-*N*-acetylhexosaminidase, molecular modeling, site-directed mutant, substrate specificity.

β-*N*-Acetylhexosaminidases (GH20; EC 3.2.1.52) are exo-glycosidases with a dual activity towards both *N*-acetylglucosamine (GlcNAc) and *N*-acetylgalactosamine (GalNAc) [1]. This substrate promiscuity is a hurdle in the selective **enzymatic synthesis** of *N*-acetylhexosamine oligosaccharides combining both GlcNAc and GalNAc units. GalNAc-terminated *N*-acetylhexosamines may be used as tailored lectin ligands in biomedicine [2]. Notably, there are no native GalNAc-ylating enzymes in nature.

We present here genetic engineering of a **synthetically potent** and **promiscuous β-*N*-acetylhexosaminidase from *Talaromyces flavus*** (*Tf*Hex), which, as a WT, exhibits a GalNAase/GlcNAcase ratio of 1.2. By molecular modeling, we identified crucial amino acid residues responsible for its GalNAcase/ GlcNAcase selectivity. **Six site-directed mutants** were prepared, heterologously expressed in *Pichia pastoris*, purified and characterized. As a result, novel engineered enzymes with a **high specificity** for either GalNAc or GlcNAc substrates were afforded. The favorable properties of the WT *Tf*Hex, mainly the **transglycosylation potential** and **tolerance to substrate functionalization**, were preserved. The substrate selectivity and transglycosylation yield were further accentuated by **reaction medium engineering**. The new toolbox of selective and synthetically capable enzymes was applied in the preparation of **tailored bioactive *N*-acetylhexosamines**.

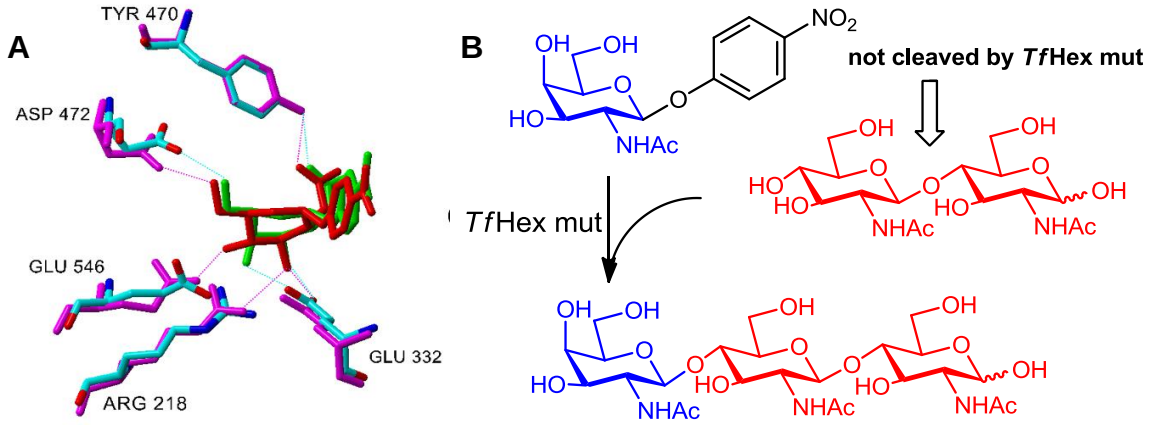

Figure 1. A, *Tf*Hex active site with docked *p*NP-GalNAc (green) and *p*NP-GlcNAc (red) after 10 ns of molecular dynamics simulation. **B**, Synthetic reaction yielding *N*-acetylhexosamines.
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Shifting the nucleotide diphosphate sugar promiscuity of glycosyltransferase C towards dTDP-fucose for fucosylation of polyphenols

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Keywords: Glycosyltransferase, fucosylation, enzyme engineering, biocatalysis.

Fucose is a sugar that can be found in several organisms including mammals, where it plays an important role in numerous mechanisms such as e.g. cell-cell-signaling [1]. Polyphenols are naturally occurring, secondary plant metabolites, that are known to possess an abundance of health benefits. Therefore, the capability of efficient polyphenol fucosylation could give rise to novel compounds, which are of interest for cosmo- or pharmaceutical applications.

In 2013 Rabausch *et al*. identified the novel glycosyltransferase C (GtfC), belonging to the GT1 family, in a functional metagenome screening of the river Elbe sediment. In whole cell bioconversion experiments it was shown that GtfC is capable of transferring hexose moieties from dTDP sugar donor nucleotides onto different polyphenols, e.g. quercetin, kaempferol and naringenin.[2]

GtfC mainly utilizes dTDP-rhamnose as sugar donor, which is synthesized in *E. coli* by the *rml* operon (Figure 1). Interestingly GtfC not only transfers dTDP-rhamnose, but also intermediates of its metabolic pathway in trace amounts.

To take advantage of this observed NDP-sugar promiscuity a plasmid based, synthetic pathway was created using *E. coli* - native *rmlAB* combined with the *fcd* gene of *Anoxybacillus tepidamans*, leading to the formation of dTDP-fucose. HPLC/MS spectra of *E. coli* whole cell biotransformations indicate the GtfC mediated transfer of fucose to naringenin in trace amounts.

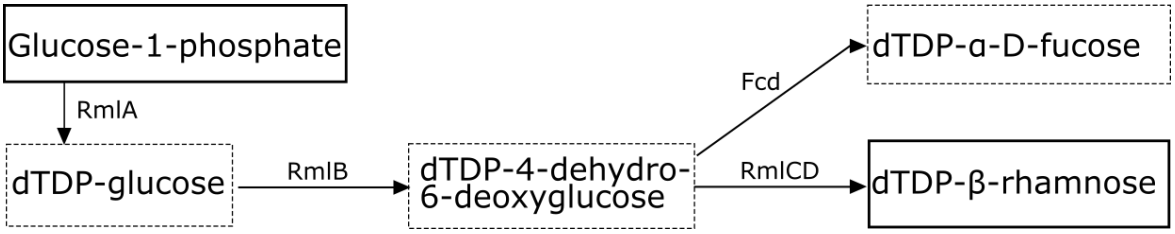

Figure 1. The metabolic pathway of the *rmlABCD* gene cluster, yielding dTDP-β-rhamnose from Glucose-1-phosphate. A replacement of the *rmlCD* genes with the *fcd* gene from *A. tepidamans* leads to the formation of dTDP-α-D-fucose. Dashed boxes indicate the detection of trace amounts transferred by GtfC.

Based on these findings, it is attempted to direct the specificity of GtfC towards the transfer of fucose onto polyphenols by random and site directed mutagenesis approaches.

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Systematic engineering of *Bacillus* sp. MN chitosanase for altered subsite specificity

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Keywords: Protein engineering, Chitosan oligosaccharides, Chitosanase

Chitosans are a family of heteropolymers consisting of β-1,4-linked *N*-acetyl-D-glucosamine (A) and D-glucosamine (D) units. They are characterized by their fraction of acetylation (FA), degree of polymerisation (DP), and also pattern of acetylation (PA). In order to study the influence of these parameters on the biological activity of chitosans, it is extremely important to efficiently produce highly defined partially acetylated chitosan oligosaccharides (paCOS). A variety of chitinases, chitosanases, and chitin deacetylases varying in substrate specificity can be employed for this task [1].

One of these enzymes is *Bacillus* sp. MN chitosanase (CSN-MN) which almost exclusively produces paCOS with the dyad DD at the newly formed reducing end. The active site of CSN-MN consists of at least six subsites ranging from (-3) to (+3), with hydrolysis occurring between (-1) and (+1). Subsites (-2) and (-1) were shown to be highly specific for D, while subsites (+1) and (+2) can accept dyads of DA or AD [2].

In order to alter subsite specificity of CSN-MN and therefore broaden the range of defined paCOS that can be produced enzymatically, 15 amino acids were selected based on their position in the substrate binding cleft of CSN-MN, and systematically substituted with the remaining 19 proteinogenic amino acids. Muteins were screened using microtiter plate-based mass spectrometric product analysis, and promising muteins were used for the enzymatic hydrolysis of well-defined chitosan polymers, followed by detailed analysis via MS^n of the paCOS produced, to identify muteins exhibiting changed subsite specificity compared to the wildtype.

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Extending the chemical space of CAZymes: how and why?

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Keywords: non-canonical amino acid, protein engineering, (bio)orthogonality, artificial enzyme.

Standard protein engineering usually involves the substitution of one or more amino acids by other amino acids chosen from the remaining nineteen common alternatives. While this approach has a proven track record, it is nevertheless extremely limited by the chemical space offered by the 20 canonical amino acids.

Over the last 25 years, techniques have been developed to site specifically introduce non-canonical amino acids (ncAA) into proteins, while using the existing genetic code [1,2]. Advantageously, these approaches provide a means to explore chemical space, create new catalytic opportunities and perform bioorthogonal conjugate reactions or assemblies.

Despite the increasing use of ncAA, this approach has rarely been used to engineer carbohydrate-active enzymes (CAZymes). In work aimed at extending the chemical space of CAZymes, we are studying the production of nCAA-bearing glycoside hydrolases and carbohydrate-binding modules [3]. In this presentation, we will describe current progress towards this goal, focussing both on the methodological aspects and on prospects for the future creation of artificial enzymes displaying new functions or architectures.

Acknowledgments. This research was supported by the pre-competitive program of Toulouse White Biotechnology (TWB), the Institut National de la Recherche Agronomique (INRA) and the 3BCAR Carnot Institute, through the project INSEREE (2015-2018), the ANS CHIMZYM (2016 & 2017) and the project i-INSEREE (2018-2019) respectively. The iGEM Toulouse INSA-UPS team 2018 (http://2018.igem.org/Team:Toulouse-INSA-UPS) is composed of G. Bordes, A. Pelus, Y. Bouchiba, C. Burnard, J. Delhomme, J. Pérochon, M. Toanen, A. Verdier, C. Wagner, S. Barbe, B. Enjalbert, J. Esque, M.-P. Escudié, R. Fauré, M. Guionnet, A. Henras, S. Heux, P. Millard, C. Montanier, and Y. Romeo.

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Improvements of fungal cellulolytic β-glucosidase D2-BGL via directed evolution

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Keywords: GHF3 β-glucosidase, lignocellulosic biomass, directed evolution, enzyme engineering

The new generation biofuels derived from lignocellulosic biomass are an important sustainable source of liquid energy. Cellulose released from biomass after physical and chemical pretreatments is converted to fermentable sugars by enzymatic reactions. Cellulases from cellulolytic microorganisms are commonly used in synergism as biocatalysts for cellulose hydrolysis. One of the cellulolytic enzymes, β-glucosidase (BGL), is essential for the hydrolysis of dimeric cellobiose to glucose. β-Glucosidase D2-BGL is a glycoside hydrolase (GH) family 3 enzyme isolated from the filamentous fungus *Chaetomella raphigera*. Using a *Pichia pastoris* expression system, the production of D2-BGL has been increased by six-fold with improvements in fermentation technology. D2-BGL has much higher substrate affinity (lower K_M value) than the commercial enzyme Novozyme 188, and it is able to work synergistically with *Trichoderma reesei* cellulases for efficient hydrolysis of acid-pretreated rice straws and sugarcane bagasse. To further improve the productivity and catalytic efficiency of D2-BGL, we performed error-prone PCR-based mutagenesis and selected 6 mutants among more than 30,000 transformants. One mutant with a mutation in the alpha secretory peptide resulted in 51% increased enzyme production, while five others carrying mutations in D2-BGL coding sequence exhibited up to 33% increase in specific activity than the wild-type D2-BGL. The productivity of D2-BGL in *Pichia* was further enhanced by 2-fold in mutants generated from a second round random mutagenesis. A new *Pichia* strain that yields higher quantity of β-glucosidase with higher specific activity will have potential applications in the industrial production of cellulosic biofuels

Computational strategy for protein design based on structure-dynamics-activity relationship insights: GH11 Xylanases as a case study

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Keywords: Xylanases GH11, molecular dynamics simulations, free energy calculations, protein design.

Enzyme internal dynamics is intimately related to molecular function [1]. A better understanding of the tight relationships between the structure, the dynamics and the properties/activities of enzymes is crucial for guiding the engineering of tailor-made enzymes with improved catalytic efficiency and/or stability in various extreme conditions for a large range of biotechnology applications. Endo-β-1,4-xylanases from Glycoside Hydrolase family 11 (GH11) are of prime interest for use in white biotechnology, biorefinery as well as bread making and biobleaching of kraft pulps [2]. However these enzymes do not always display the properties required for their use in bioprocesses [3].

In this study, four GH11 xylanases from different sources and displaying distinct catalytic efficiencies and thermosabilities were studied; a highly active xylanase from *Neocallimastix patriciarum*, a xylanase from *Thermobacillus xylanilyticus*, an environmentally isolated thermophilic xylanase and its hyperthermostable variant. Large-scale intramolecular flexibility and conformational rearrangements of free enzymes and in interaction with substrate were investigated by extensive brute-force molecular dynamics simulations at different temperatures, combined with several post-processing techniques, including enzyme-substrate affinity computation (MM/GBSA[4]), principal component analysis (PCA) as well as free energy landscape[5] and dynamic cross correlation analyzes. Our results provide a detailed picture at atomic level of the molecular motions occurring during different steps of catalytic process and highlight functionally-relevant molecular regions of xylanases. Comparison of dynamics behavior of the four studied xylanases led to the identification of key structural motifs involved in substrate recognition and molecular flexibility which modulate xylanase activity and stability. The developed *in silico* approach is highly valuable to pinpoint hot spot amino acid residues with the aim to alter enzyme activity and stability, thus being of the crucial importance for protein engineering.

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Screening assay for directed evolution of chitin deacetylases

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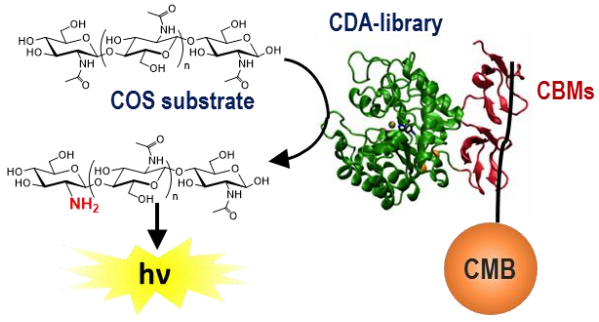
Keywords: chitin deacetylases; directed evolution; HTS, chitooligosaccharides,

Deacetylation of chitin and chitooligosaccharides (COS) renders chitosans (with variable degrees of acetylation (DA)) and partially deacetylated oligosaccharides (paCOS) with considerable industrial interest as biocompatible, biodegradable, and non-toxic functional materials with a wide variety of biotechnological and biomedical applications. The degree of acetylation (DA) strongly influences the physicochemical and biological properties of chitosans and paCOS, but the role of the pattern of acetylation (PA), which defines the distribution of charged D-glucosamine ($GlcNH_2$) residues along the N-acetyl-D-glucosamine (GlcNAc) polymeric/oligomeric chain, on the specific interactions with biological receptors and target structures remains largely unknown.

Chitin de-N-acetylases (CDAs) catalyze the hydrolysis of the acetamido group in GlcNAc residues of chitin, chitosan, and COS. The deacetylation pattern exhibited by CDAs and related carbohydrate esterase (CE4) enzymes active on COS is diverse, some being specific for a single position, others showing multiple attack mechanisms. After solving the first 3D structure of a *Vibrio cholera* CDA in complex with substrates, we are addressing structural and biochemical studies with the goal of understanding the determinants of substrate specificity of CDAs and related CE4 enzymes active on COS [1-4]. The search of new enzymes and the engineering of substrate specificity, either by structure-guided and directed evolution approaches will extend the toolbox of selective enzymes for the biotechnological production of tailored and sequence-defined COS to evaluate their biological functions and develop novel biotech and biomedical applications.

Here, we report the design and development of a HTS assay in micro-titer plate format using a medium scale robotic platform for the screening of directed evolution libraries of CDAs and other CE4 enzymes active on COS and its proof-of-concept application to the engineering of substrate specificity of *Vc*CDA. The assay is based on fusion of the target CE4 catalytic domain to be evolved to a chitin binding module (CBM), capture of the expressed proteins from cell-free extracts with chitin-coated magnetic beads (CMB), and evaluation of the deacetylase activity of the immobilized enzyme variants on COS substrates by monitoring product formation with a coupled assay leading to a fluores-cence readout [5].



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Rational protein design of *Bacillus* sp. MN chitosanase for altered substrate binding and large-scale production of specific chitosan oligomers

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Keywords: Protein engineering, Chitosan oligosaccharides, Chitosanase

Chitosan oligosaccharides (COS) consisting of β-1,4-linked *N*-acetyl-D-glucosamine and D-glucosamine units have various potential applications in agriculture, biomedicine, and pharmaceutics due to their suitable bioactivities. The bioactivities have been shown and proposed to be dependent on not only the molecules´ degree of polymerization (DP) and acetylation (DA), but also on their pattern of acetylation (PA). To elucidate molecular structure-function relationships, the effective production of defined paCOS is crucial, both for fundamental research and for developing chitosan-based applications. The best way to produce defined paCOS is partial enzymatic deacetylation of chitin oligomers or partial enzymatic *N*-acetylation of glucosamine oligomers using regioselective chitin deacetylases in direct or reverse mode of action, respectively. For example, all 14 possible variants of tetrameric paCOS can thus be produced from chitin tetramer (A_4) and the fully deacetylated tetramer (D_4) [1]. This approach requires production of abundant amounts of the two tetramers, best using chitinases or chitosanases, but this is challenging because the tetramers are generally hydrolyzed further by these enzymes. We here describe rational protein engineering on the extensively studied GH 8 chitosanase CSN from *Bacillus* sp. MN [2, 3] to abolish tetramer hydrolysis. By specifically targeting residues with a predicted function in substrate binding, we created new muteins incapable of efficiently hydrolyzing the fully deacetylated tetramer D_4, and we were able to demonstrate efficient large-scale production of D_4 with an altered version of CSN.

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Transglycosylation activity of glycosynthase-type mutants of β-*N*-acetylhexosaminidase from *Talaromyces flavus*

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Keywords: β-*N*-acetylhexosaminidase, glycosynthase, mutation, transglycosylation.

The β-*N*-acetylhexosaminidase (CAZy GH20, EC 3.2.1.52) from the filamentous fungus *Talaromyces flavus* features unique substrate flexibility and remarkable synthetic ability. Generally, the yields of the transglycosylation reactions catalysed by glycosidases are significantly lowered by the parallel hydrolysis of the glycosidic bonds both of the substrate and products. We have recently described the design and universal utility of transglycosidase mutants of *T. flavus* β-*N*-acetylhexosaminidase featuring diminished hydrolytic activity and high transglycosylation activity, which were achieved by mutating the oxazolinium transition state intermediate stabilizing residue Tyr470.

Glycosynthases have been established ca 20 years ago as the mutant glycosidases with their catalytic nucleophile (Asp/Glu) changed for a small and neutral residue, typically glycine or alanine. These synthetic enzymes have lost their hydrolytic activity, however, they are able to transfer glycosyl moieties from activated donors (fluorides) of the opposite configuration with outstanding yields. In this study, we aimed at the preparation of the first glycosynthases from the GH20 enzyme employing the substrate-assisted catalysis proceeding *via* the oxazolinium intermediate. **Seven mutants of the catalytic nucleophile Asp370 were designed** and expressed in *Pichia pastoris*; moreover, two of the prepared variants comprised **double mutation combined with the previously successful mutation of Tyr470**. Most of the obtained enzyme variants practically lost their hydrolytic activity towards the standard substrate *p*NP-GlcNAc, moreover, the mutants were not active with the proposed glycosynthase donor GlcNAc-α-F as well. After careful optimization of reaction conditions, we found that the **mutant enzymes retained significantly decreased activity towards the standard β-substrate *p*NP-GlcNAc**, which can even be **preferably transferred in a transglycosylation reaction**, yielding substantial amounts of *p*NP-chitobiose and *p*NP-chitotriose in some cases.

This is the **first report on the functional mutants of the active-site nucleophilic residue of the GH20 β-*N*-acetylhexosaminidase**, which shows that the enzymes employing the substate-assisted catalytic mechanism **do not follow the general rules for the preparation of a glycosynthase** from a β-retaining glycosidase. Molecular modelling revealed the intact interactions between the mutant enzymes and the oxazolinium transition state, while the GlcNAc-α-F could not be accommodated in the active site with a stable position enabling transglycosylation to proceed.

Acknowledgements: Support from Czech Science Foundation project No. 18-01163S and Czech Ministry of Education project LTC18041 is acknowledged.

Transglycosidases engineered from the β-*N*-acetylhexosaminidase from *Aspergillus oryzae*

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Keywords: β-*N*-acetylhexosaminidase, mutation, transglycosylation

The β-*N*-acetylhexosaminidase (EC 3.2.1.52, GH20) from the filamentous fungus *Aspergillus oryzae* (*Ao*Hex) has previously been shown to possess a remarkable synthetic ability. However, in the wild-type enzyme the yields of the transglycosylation reactions are significantly lowered by the concurrent hydrolysis of the glycosidic bonds of both the substrate and products. To overcome this problem, **mutant variants of *A. oryzae* β-*N*-acetylhexosaminidase** were designed by molecular modelling based on the recently published crystal structure of this enzyme, aiming at diminishing its hydrolytic activity and retaining transglycosylation activity at once, e. g. at the preparation of a **transglycosidase**. In this work we focused on the **aglycon-binding site**, where we aim at increasing its hydrophobicity by introducing tryptophan residues in order to **improve binding of the acceptor sugar in the transglycosylation reaction**.

Two new mutant variants of *Ao*Hex (**F453W**, **V306W**; Fig. 1), which were predicted to feature lower hydrolytic activity and increased ability to transglycosylate substrates in natural β-configuration, were prepared and characterized. The mutant β-*N*-acetylhexosaminidases were heterologously expressed in methylotrophic yeast *Pichia pastoris* and purified from its culture media in a single step. The transglycosylation activity of the recombinant enzymes was tested with *p*NP-GlcNAc as a substrate. Both of the prepared enzymes show **decreased hydrolytic activity and catalyze transglycosylation reactions with higher yields than the WT enzyme**. This is the first report on the functional mutants of the aglycon-binding residues of the GH20 β-*N*-acetylhexosaminidase, which opens **new routes to the preparation of effective GH20 transglycosidases** applicable in the synthesis of bioactive chitooligomers.

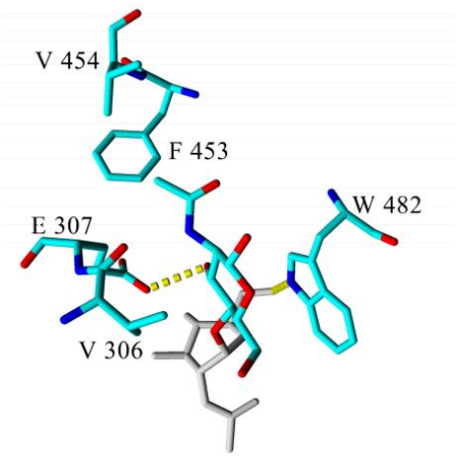

Figure 1. Active site amino acids of *Ao*Hex within 3 Å distance from the aglycon (reducing-end) part of docked chitobiose, which is shown in colors by element colors, the non-reducing GlcNAc moiety of chitobiose is shown in gray. Hydrogen bonds with visualized residues are depicted in yellow interrupted lines.

Support from Czech Ministry of Education project LTC18003 is acknowledged.

VersaTile: A high-throughput DNA assembly method for the rapid construction and evaluation of cellulosome components

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Keywords: Designer cellulosomes, lignocellulose, galactomannan, xyloglucan

We have developed a DNA shuffling method (VersaTile) for the assembly of non-homologous coding sequences, which enables us to create modular proteins at a high rate. The method involves the construction of a repository of 'tiles' or modules that one wants to shuffle, followed by the assembly of any combination of these tiles. This method was implemented on the principle of 'designer cellulosomes', which are the synthetic version of one of natures most advanced modular proteins, cellulosomes.

Designer cellulosomes are multi-enzyme complexes composed of two complementary structural modules. A large non-catalytic backbone molecule, the scaffoldin, comprises several cohesin modules, whereas various enzymatic subunits are composed of a dockerin and a catalytic module. The cohesins selectively integrate specific cellulolytic enzymes into the complex due to the high affinity cohesin-dockerin interaction. Since all catalytic modules with complementary functions are close to each other, their synergy is enhanced. On top of that, the protein engineer has control over the composition and arrangement of selected enzymes, enabling the customization of designer cellulosomes for specific lignocellulosic sources

At the moment, the preliminary DNA work is viewed as the major bottleneck in the production process, which explains why most studies have focused on the production and analysis of one or a few designer cellulosomes at a time. However, performed research has also shown that there is a high number of variables influencing the performance of designer cellulosomes. For example, changing the relative position of different docking enzymes on the scaffoldin can have a major effect. In theory, each specific collection of enzymes requires optimization in order to assemble the most optimal set of docking enzymes on the ideal scaffoldin. This calls for a quick and easy assembly method.

Since both structural modules are highly modular, the concept of designer cellulosome production strongly agrees with the VersaTile approach. Practically, we have selected galactomannan and xyloglucan, two major hemicellulose fractions that have not been covered in the field of designer cellulosome research yet, as substrates for two separate case studies. We created a tile repository containing a range of cohesins, dockerins, CBMs, linkers and the essential enzymes for the full degradation of each of these substrates and were able to investigate the effect of different parameters (dockerin type, dockerin position, type of linker) on the performance of the constructed designer cellulosomes.

In sum, we now have a high-throughput method for the rapid and convenient construction of designer cellulosomes. This allows the essential preliminary assays that are needed to achieve the most efficient multi-enzyme complex, which is a great step forward in the field.

High-throughput enzyme engineering for commercial-scale production of natural products

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Keywords: High-throughput enzyme engineering

The natural products world is unparalleled in its molecular diversity and wide application space. There are however numerous challenges associated with realizing the full potential of these molecules. Amyris has produced eight different molecules through fermentation at commercial scale. This track record is due to investment in advanced tools for strain engineering, high throughput screening, analytics, and bioinformatics. An integrated pipeline encompassing these tools has enabled Amyris to rapidly accelerate the engineering cycle and reduce the number of design-build-test iterations needed for microbial production of any natural product. This infrastructure is now being leveraged for high-throughput enzyme screening and mutagenesis, enabling greater access to natural products and their derivatives. Further, the application of our massive screening infrastructure to enzyme libraries would not be possible without equally sophisticated statistical models and data analysis tools. Scientists at Amyris are accessing ever greater portions of the enzyme sequence space to improve specificity and activity – ultimately enabling sustainable industrial-scale production of natural products. This poster will describe how each aspect of the enzyme engineering pipeline has led to rapid and high-quality screening of hundreds of thousands of mutants for multiple enzymes.

Increased trans-glycosylation activity of hexosaminidases for synthesis of human milk oligosaccharides

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Keywords: hexosaminidase, transglycosylation, protein engineering, human milk oligosaccharides.

The composition of human breast milk differs significantly from that of bovine milk, which is used for infant formula production. Especially the presence of human milk oligosaccharides (HMOs) contributes health and development promoting features for newborn infants. Nevertheless, not all newborns and especially premature infants can be breast-fed for different reasons. With respect to this we are developing new enzymatic routes for synthesis of HMOs, which can be used as functional ingredient for infant formula.

Previously, we identified two candidate GH20 hexosaminidases from a metagenomic library, which were able to synthesize the basic HMO backbone structure, lacto-*N*-triose II, from chitobiose and lactose by trans-glycosylation [1]. Since the yields using these enzymes were low, we wanted to improve their trans-glycosylation activity to increase their applicability for a feasible process. To keep the screening effort low, a rational design approach was followed. Peptide pattern recognition (PPR) analysis [2] was performed on the whole GH20 CAZy family (approx. 3000 sequences) to identify other enzymes with potential trans-glycosylation activity based on relatedness. By phylogenetic analysis of the group containing the two known enzymes (approx. 1000 sequences) and subsequent alignment of the closely related sequences, a loop insertion close to the active site was identified [3]. Homology modelling revealed that introduction of this loop structure into hex1 and hex2 would lead to a significantly narrower active site and therefore contribute to exclusion of water from the active site, which is a well-known strategy to increase trans-glycosylation activity. The proposed loop mutants were expressed, purified and characterized for trans-glycosylation activity. While loop insertion did not improve hex2, it was found that for hex1 three out of four loop mutants showed an up to 9-fold improved trans-glycosylation activity compared to the wild-type [3]. In conclusion, we succeeded in engineering an enzyme towards increased trans-glycosylation activity using a custom-made rational approach utilizing available sequence analysis methods.

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Transfucosylation by and engineering of GH29 α-L-fucosidases for production of human milk oligosaccharides

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Keywords: α-L-fucosidase, transglycosylation, protein engineering, human milk oligosaccharides.

Human milk oligosaccharides (HMOs) are crucial for infant health and development, but absent from bovine milk and thus from infant formula. Among them, fucosylated HMOs are the most abundant ones. Glycosidase-catalysed transglycosylation is one way of producing HMOs from naturally available substrates. Retaining α-L-fucosidases from GH29 can catalyse transfucosylation at optimized conditions. However, substrate specificity and regioselectivity may vary. In a recent study [1], we expressed and characterized seven different α-L-fucosidases in terms of substrate specificity, thermal stability and transfucosylation capacity. All seven α-L-fucosidases were able to catalyze transfucosylation from 2'-fucosyllactose (2'FL) or 3-fucosyllactose (3FL) to lacto-*N*-tetraose (LNT) to yield the more complex HMO structures lacto-*N*-fucopentaose (LNFP) I and II with yields ranging from 10% to 40%, but with varying degree of regioselectivity [1].

Natural fucosyl donor substrates beyond HMOs 2'FL and 3FL are scarce. This study describes the first example of an α-L-fucosidase (*Fg*FCO1 from *Fusarium graminearum*) able to catalyze transfucosylation from fucosylated xyloglucan extracted from citrus peel – an abundant agroindustrial side stream – to lactose to yield a true HMO structure, 2'FL [1].

Another study included two α-1,3/4-L-fucosidases from GH29 subfamily B, namely *Bb*AfcB from *Bifidobacterium bifidum* and *Cp*Afc2 from *Clostridium perfringens*. While *Cp*Afc2 efficiently catalyzed transglycosylation, *Bb*AfcB exhibited significant hydrolytic activity impairing its transfucosylation yields. Comparing homology models of *Bb*AfcB and *Cp*Afc2 revealed that they appear similar in terms of active site structure and substrate-interacting residues, but an α-helical loop on the side of the active site constituted a major difference between them. It was hypothesized that this loop plays a role in shielding the active site from the aqueous environment, which takes part in substrate and product hydrolysis. Exchanging the 23 amino acids long α-helical loop close to the active site of *Bb*AfcB with the corresponding 17-amino acid α-helical loop of *Cp*Afc2 resulted in almost complete abolishment of the hydrolytic activity on 3FL (6000 times lower than WT *Bb*AfcB), while the transfucosylation activity was lowered only one order of magnitude. In turn, the loop engineering resulted in a regioselective α-1,3/4-L-fucosidase with transfucosylation activity reaching molar yields of LNFP II of 39 ± 2% and negligible product hydrolysis. This was almost 3 times higher than the yield obtained with WT *Bb*AfcB (14 ± 0.3%) and comparable to that obtained with *Cp*Afc2 (50 ± 8%) [2].

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Co-immobilization of a biocatalytic oxidation-amination pathway through autocatalytic association of proteins

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Keywords: Enzyme immobilization, galactose oxidase, omega-transaminase, fusion proteins.

A recent study from our group involves the production of aminocarbohydrates *via* a two-enzyme oxidation-transamination pathway using a galactose oxidase (variant M8) and an omega-transaminase (Uniprot: P0CS93 and Q7NWG4) [1,2]. The conversion of the two-step pathway is likely to be restricted by formation of side products from a reactive aldehyde intermediate. Through co-immobilization, we aim bring the two enzymes within close proximity to each other, and to optimize the ratio of the two enzyme activities, in order to control the concentration of the reactive aldehyde intermediate, mimimize by-product formation, and therefore improve the overall conversion of the pathway. The co-immobilization is based on the autocatalytic association of two protein domains (named Jo and In) derived from the pilus adhesin found in *Streptococcus pneumoniae*, developed by Bonnet et al. (2018) [3]. The JoIn system acts as a rigid spacer system, limiting interactions of an enzyme with the solid support, and holds the catalytic domain in a fully solvent accessible orientation [4]. Therefore, the JoIn system has a low impact on enzyme activity and, given the near-quantitative immobilization efficiency [4], this method provides an ideal tool for studying activity ratios, as well as the effects of proximity of the enzymes included in the pathway on the conversion of the two-step reaction.

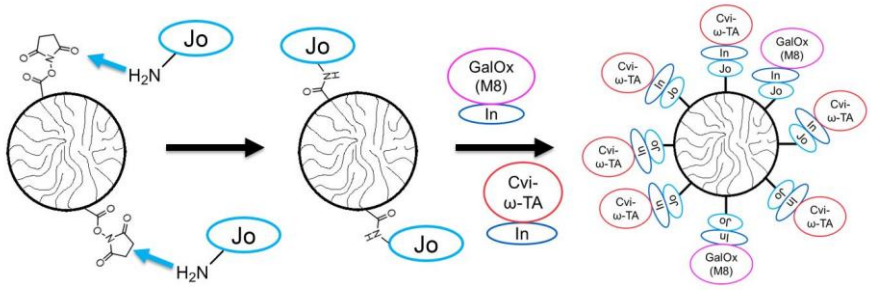

Figure 1. Mechanism of binding Jo to NHS-ester functionalized paramagnetic beads, and the autocatalytic association of the Enzyme-In fusion proteins to Jo-coated beads.

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Imidazolium-labeled glycosides for the characterisation of enzymatic function during plant biomass degradation

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Keywords: Cellulase, LPMO, Mass spectrometry, Substrate

Cellulose is the most abundant biopolymer on Earth.. Enzymes involved in cellulose modification and degradation are ideal candidates for producing high value materials and chemicals from renewable biomass, thus contributing to a future bio-based economy.
Due to their evident economic importance, there is a strong demand for well-characterised, effective LPMOs (lytic polysaccharide monooxygenases) and cellulases. To assess the function of uncharacterized enzymes on cellulose we are developing substrates that are ideally suited for microliter scale reactions followed by quick, inexpensive, information rich and highly sensitive MALDI-ToF mass spectrometry analysis.

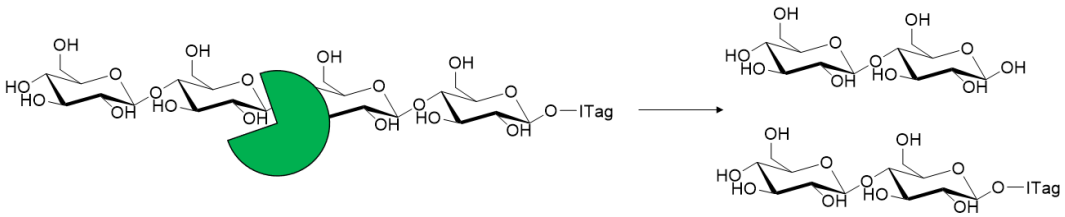

Figure 1. ITag-glucose oligosaccharide acting as a substrate for cellulase and the subsequent degradation products produced.

Ionic liquid-based Imidazolium tags (ITag-) ionise well in MS and thus vastly increase the signal of their conjugate [1]. We discovered an enzyme able to polymerise glucose onto ITag-glucose, to form oligosaccharides with a degree of polymerization of up to 9. We show that these oligosaccharides are substrates of both oxidative and hydrolytic cellulolytic enzymes (Fig. 1). Sensitive detection of reaction products via MALDI-TOF MS enables identification of substrate / product range, and type of oxidative mechanism.
The flexibility of enzymatic synthesis of these substrates enables potential incorporation of modified building blocks at both termini of the oligosaccharides. In proof of principle studies the non-reducing termini of oligosaccharides were capped by addition of galactose and subsequent chemo-enzymatic derivatisation. This strategy blocks any exo-acting activity but allows endo-acting cellulases to degrade the internal oligosaccharide chain.
In conclusion, we demonstrate flexible chemo-enzymatic synthesis of substrates for sensitive, MS based detection of oxidative and hydrolytic cellulosic enzyme activity, which is more broadly applicable to glyco-enzyme characterisation.

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Novel enzymes to obtain bioactive oligosaccharides from a marine-derived complex exopolysaccharide

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Keywords: Marine bacteria, exopolysaccharides, depolymerization, sulfatase.

Many marine bacteria produce structurally-unique exopolysaccharides (EPS) whose features are close to animal-derived bioactive glycosaminoglycans. This class of compounds, involved in numerous cellular processes in both healthy and diseased tissues, is studied today to bring therapeutic responses. Marine-derived EPS studied at Ifremer exhibit exploitable biological activities in the domains of the cosmetic and the human health with or without subsequent structural modifications. In particular, the chemically depolymerized and over-sulfated by-products of the EPS GY785 produced by the bacterium *Alteromonas infernus* [1,2] showed an inhibitory effect on the metastatic process of tumor cells in an osteosarcoma animal model [3].

Enzymes are advantageous tools to develop more specific and better controlled processes in order to obtain from native EPS bioactive derivatives with well-defined structures and sizes. During preliminary studies, it has been shown that no commercial enzyme among 30 tested was active on the EPS GY785. However, *A. infernus* bacterium which biosynthesizes the EPS, also produces, in certain conditions, enzymes capable of generating oligosaccharides, probably as a mixture [4]. Oligosaccharide production but also characterization of enzyme activities have been investigated.

In addition to the added value of their biological activity, finely characterized oligosaccharides are useful tools as standards for both structural analysis of complex polysaccharides and understanding of their structure-function relationships.

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Stereocontrolled chemo-enzymatic conversion of CO_2

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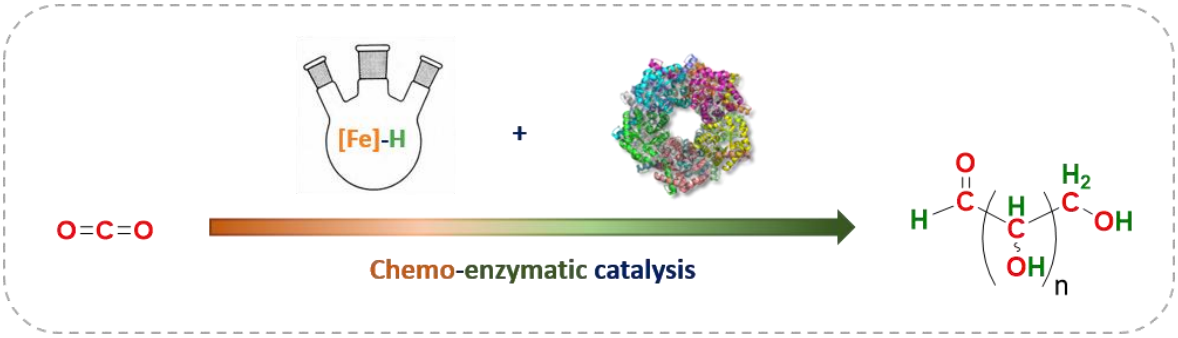
Keywords: CO_2, carbohydrate, formaldehyde, stereocontrol.

CO_2 is a crucial molecule for life on Earth. Indeed, it represents an abundant source of carbon and an energy carrier for living organisms. Through the Calvin Cycle, or "Dark Cycle", CO_2 is converted into carbohydrates, the most abundant and diversified class of biomolecules on Earth. They are involved in many biological processes and consequently represent an essential class of therapeutic targets. Carbohydrates are mainly synthesized by transformation of readily available sugars extracted from the biomass or from fossil resources. However, synthetic access to well-defined, rare and stereocontrolled carbohydrates from a renewable, achiral, and simple carbon source such as CO_2 is a fundamental and very challenging process.



Scheme 1. Chemo-enzymatic cascade for CO_2 conversion into carbohydrates.

On one hand, iron hydride complexes have proved their high capabilities toward CO_2 reduction [1]. On another hand, enzymes have successfully demonstrated their ability to convert C1 building blocks into stereocontrolled and well-defined products, such as carbohydrates [2] (Scheme 1). We wish to present how the combination of chemo and enzymatic -hybrid- catalysis enables to open a new synthetic pathway for carbohydrates synthesis from CO_2 as the only source of carbon.

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Protein engineering of Lacto-*N*-biose from *B. bifidum* for HMOs production

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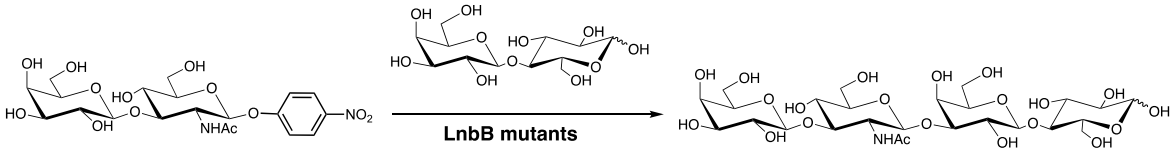
Keywords: Human milk oligosaccharides, HMOs, transglycosylation, protein engineering.

Human milk is considered the gold standard for infant nutrition; it is a complex mixture that besides providing complete nutrition to the infant, it delivers essential biomolecules. Human milk oligosaccharides (HMO) are a group of glycans that provide essential biological functions such as immune modulators, prebiotics, and nutrients for neonatal brain development [1].
Bifidobacterium bifidum present in the gut's infant produces lacto-*N*-biosidase (LnbB), which is involved in the catabolism of HMOs. LnbB belongs to the GH20 family *N*-acetylhexosaminidases that operate by a retaining substrate-assisted catalytic mechanism [2][3]. LnbB catalyzes the hydrolysis of the tetrasaccharide lacto-*N*-tetraose to lacto-*N*-biose and lactose [4]. Our structural-functional analysis has revealed the multi-domain organization of the enzyme and the important residues for its hydrolytic activity [5].

The aim of this work is the lacto-*N*-tetraose (core 1 HMO) production, for this, here we report the engineering of LnbB following a semi-rational approach [6]. To obtain a transglycosylating enzyme we are targeting the conserved residues located in the negative subsites of the binding site. A number of mutants are characterized, the hydrolytic activity of the enzymes was tested on LNB-pNP (lacto-*N*-biose pNP) and the transglycosylation activity was studied using lactose as an acceptor and LNB-pNP as a donor. Current results on the engineering of an efficient transglycosylating biocatalyst will be reported.



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Is the GBE from *Butyrivibrio fibrisolvens* the perfect branching enzyme?

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Keywords: branching enzyme, starch, application, *B. fibrisolvens*

Branching enzymes are proteins active in the synthesis of either starch or glycogen and are present in all three kingdoms of life [1]. Their catalytic cycle consists of two steps. The first step, the hydrolysis, involves cleavage of an α-1,4-glycosidic linkage within a linear stretch of α-glucan. This is followed by the second step, the branching, describing the formation of an α-1,6-bond between the remaining fragment and a new linear polysaccharide chain [2]. Recently it was discovered in our group that branching enzymes were, in fact, "imperfect branchers" that sometimes failed to complete their catalytic cycle. After conducting the hydrolysis step, the enzymes did not always implement the branching step but instead transferred the remaining fragment to a water molecule, thereby forming a new short chain instead of a branch. The degree of successful branching was found to vary between branching enzymes, with the enzyme exhibiting the highest success rate being the branching enzyme from the ruminal bacterium *Butyrivibrio fibrisolvens*. Incubation of this enzyme with e.g. amylose lead to a fast increase in branching while the number of free chains (molecules) remained virtually unchanged. It should be noted that the success rate does not appear to determine the final degree of branching generated by the enzyme, but rather its overall activity. However, this ability might result in a branched product of considerably larger molecular weight than obtained from other branching enzymes. A product as such is of high industrial interest due to its slow digestion and low viscosity and thus its suitability for sport drinks and clinical nutrition. In order to gain more insight into the activity patterns of various branching enzymes, especially the one from *B. fibrisolvens*, the enzymes are overexpressed recombinantly in *E. coli* and studied on a series of different starch samples, followed by an in depth analytical analysis of the products.

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Identification and characterization of a GlcNAc de-*N*-acetylase from the hyperthermophilic archaeon *Saccharolobus solfataricus*

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Keywords: Archaea, CAZymes, *N*-acetylglucosamine, de-*N*-acetylase.

Within the crenarchaeal group of Sulfolobales, *N*-acetyl-glucosamine (GlcNAc) has been shown to be a component of exopolysaccharides (EPS), forming their biofilms [1], of the *N*-glycan decorating some proteins [2] and as biosynthetic intermediate of Glycosylphosphatidylinositol (GPI)-anchor [3]. The metabolism of GlcNAc is still poorly understood in Archaea and one approach to gaining information on that is through the identification and functional characterization of carbohydrate active enzymes (CAZymes) involved in the modification of GlcNAc. Recently, GH116 bi-functional exo-β-glucosidase/exo-β-*N*-acetyl-glucosamminidase (SSO3039), likely involved in the recycling of *N*-acetyl-glucosamine in *S. solfataricus*, has been identified and characterized [4]. By contrast, α-*N*-acetyl-glucosaminidase activities (EC 3.2.1.50), currently classified only in GH89, are unknown in Archaea. The screening of *S. solfataricus* extracts allowed the detection of a novel α-*N*-acetyl-glucosamidase activity, which has never been identified in Archaea. The mass spectrometry analysis of the purified activity showed a protein encoded by the *sso2901* gene. Interestingly, the purified recombinant enzyme, which was characterized in detail, revealed a novel de-*N*-acetylase activity specific for GlcNAc and derivatives. Thus, assays to identify an α-GlcNAcase found a GlcNAc de-*N*-acetylase instead. The α-GlcNAcase activity observed in *S. solfataricus* extracts did occur when SSO2901 was used in combination with an α-glucosidase. Furthermore, the inspection of the genomic context and the preliminary characterization of a putative glycosyltransferase immediately upstream of *sso2901* (*sso2900*) suggest the involvement of these enzymes in the GlcNAc metabolism in *S. solfataricus* [5].

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Dynamic strategies of hemicellulases production for an efficient lignocellulosic biomass fractionation

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Keywords: biomasses, deconstruction, hemicellulases, proteomics.

Lignocellulosic substrates are recalcitrant to enzymatic hydrolysis. Numerous factors such as the presence of hemicelluloses and lignins are known to be responsible of the biomass recalcitrance to fractionation. These later ones are known to impede the access of cellulases to the cellulose part (development of 2G ethanol) by forming physical barriers. A physico-chemical pretreatment is thus necessary to improve enzymes efficiency. One challenge for biorefineries is to develop enzymatic processes for hydrolysis of hemicelluloses to improve 1) the access of cellulases to cellulose and 2) the obtention of the constitutive molecules (pentoses, phenolic compounds) from these polymers. A thermophilic and hemicellulolytic bacterium (*Thermobacillus xylanilyticus*) was able to produce thermostable and hemicellulasic cocktails performant for the deconstruction of different lignocellulosic biomasses. Analyses of the genome (4Mbp representing 3900 genes predicted) allowed identifying high lignocellulolytic potential with 162 CAZYmes encoding genes that could play a role in the lignocellulose biotransformation. The aim of this study is to understand enzymatic strategies employed by the bacterium for efficient hemicelluloses fractionation. Do the lignocellulosic substrates induce a specific or similar enzymatic strategies production according to their compositions?
For this, we studied the behavior of the bacterium while growing on several substrates with contrasted chemicals and architectures (wheat bran, wheat straw, sweet corncob, barley straw pretreated or not) as well as purified hemicellulose (xylan). Multiple approaches combining growth on the various substrates, hemicellulasic activities productions and proteomic analyses of the intracellular and extracellular proteins, were performed to identify and quantify the expression level of hemicellulases produced by the bacterium. The effect of the enzymes produced during growth on the substrates were also assessed. This work has been co-funded by the European Commission (Horizon 2020 Program) under Grant agreement no. 654362 (BABET-REAL5 Project). The objective of the BABET-REAL5 project is to develop an alternative solution for the production of 2G ethanol, competitive at smaller industrial scale and therefore applicable to a large number of countries, rural areas and feedstocks such as sweet corn cob (SCC) and barley straw (BS).

Enzymatic *N*-acylation and *N*-transacylation of D-glucosamine

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Keywords: CmCBDA, *N*-acylglucosamine derivatives, *N*-acylation, *N*-transacylation.

We previously reported the isolation and characterization of a bacterial deacetylase (CmCBDA), which catalyzes selectively the hydrolysis of GlcNAc to glucosamine under mild reaction conditions[1]. This CmCBDA deacetylase was further studied and demonstrated to catalyze the *N*-acylation of unprotected glucosamine and to *N*-transacylate unprotected *N*-acetylglucosamine at ambient temperatures. A wide range of *N*-acylglucosamine derivatives bearing aliphatic chains or different functional groups suitable for further incorporation reactions were obtained in high conversion rates. Furthermore, CmCBDA catalyzed the *N*-acylation of glucosamine and was used in an enzymatic cascade for the synthesis of sialosides.

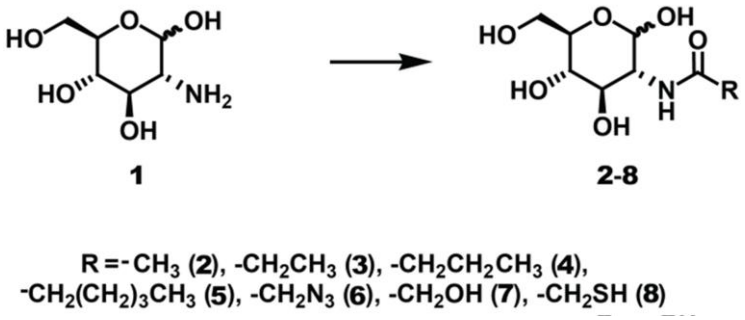

Figure 1. CmCBDA-catalyzed acylation of glucosamine **1** to provide *N*-acylglucosamines **2-8**.

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Optimization of mutation at the acid/base residue of α-glucosidase for O-α-glucosylation toward flavonoids

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Keywords: α-glucosylation, α-glucosidase mutant, α-glucosyl flavonoids

Thioglycoligase strategy using glycosidase mutant lacking their catalytic acid/base residuehas been developed for glycosylation of sugar acceptors bearing a thiol group at a proper position. The application of acid/base mutants for glycosylaton has been expanded toward selective α-O-glucosylation for compounds with an OH group with a lower pKa than water. Recently, a thermostable α-glucosidase mutant exhibits regioselective α-O-glucosylation toward various flavnoids.

In this work, it was attempt to optimization of the mutation at the acid/base residue of the thermostable α-glucosidase mutant. As the primary screening, the potential hits were selected through a screening platform based on fluorescence reduction by transglucosylation of methyumberryferone, followed by analysis of transfer product using an isoflavone as the accptor through TLC. Three additional mutants were screened from a site-saturation mutagenesis library. The best hit showed 2.5-fold imporve rate compared to the parent mutant using α-glucosyl fluoride and an isoflavone as the substrate. Interestingly, the kinetic analysis of selected mutant showed the dramatic change in the acceptor specificity. The k_{cat}/K_M for isoflavones used in the screening process as the sugar acceptor were much highly imporved compared to other flavnoids.

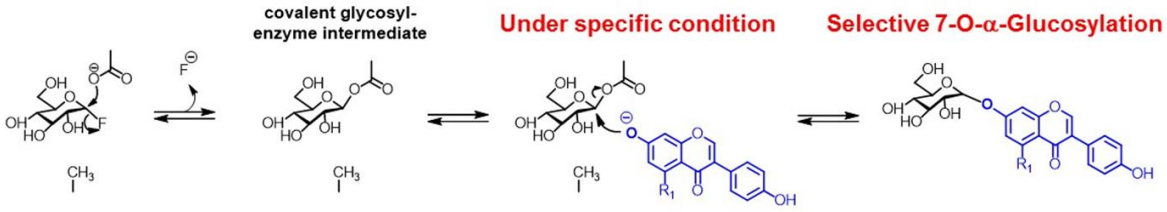

Figure 1. Scheme for Selective O-α-translgycosylation of α-glucosidase mutant.

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Thioglycosides chemoenzymatic synthesis:

S-glycosyltransferase and thioligase as efficient biocatalysts

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Keywords: thioglycosides, thioligases, *S*-glycosyltransferases, biocatalysis

S-glycosides are of particular interest because they are structural analogs of their *O*- and *N*-counterparts, but they are much more resistant to chemical and enzymatic hydrolysis. Classical organic synthesis methodologies of these S-glycosides generally require tedious protection/deprotection steps, and non-sustainable reaction conditions. To bypass these pitfalls, chemoenzymatic synthesis has emerged as an attractive approach in *S*-glycosides generation.

2 strategies have been generally used to catalyze the synthesis of thioglycosidic linkage:

- In nature, *S*-glycosyltransferases (*S*-GT) are involved in the synthesis of S-glycosides, including the well-known family of glucosinolates[1].
- The thioligase methodology[2] relies on mutated glycoside hydrolase that enable the nucleophilic attack of a thiol containing acceptor to yield the desired thioglycoside.

Both methodologies have been successfully used in our group to generate a wide diversity of thioglycosides, with potential applications as biochemical tools for enzymes studies, or as bioactive compounds in cosmetics or therapeutics. UGT74B1 from *A. thaliana* and *Dt*Gly from *D. thermophilum* have revealed interesting biocatalysts as respectively *S*-GT and thioligase, and comparison between both approaches will be presented.[3]

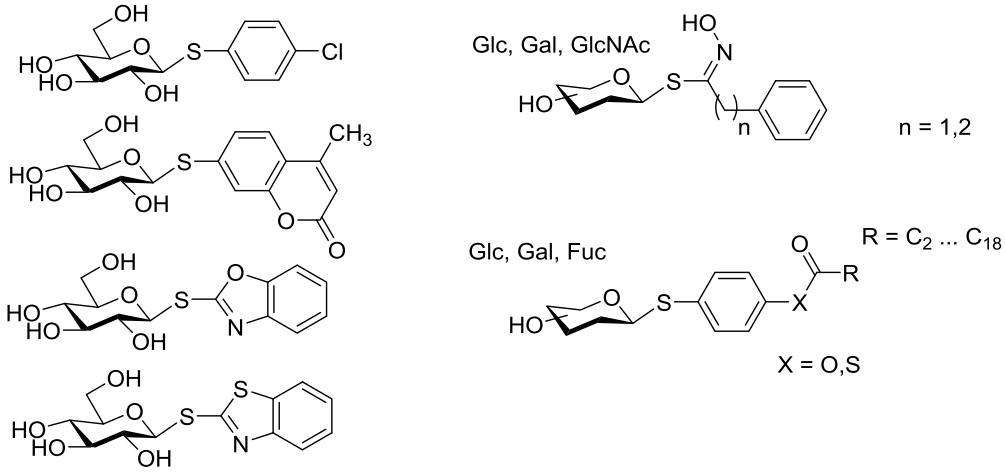

Figure 1. Thioglycosides generated using UGT74B1 and *Dt*Gly

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Diversion of the arabinofuranosidase *Ct*Araf51 for the anomeric acylation of L-arabinofuranose

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Keywords: Glycosyl hydrolase, thioligase, enzymatic acylation, acyl furanose.

Aliphatic carboxylic acid were crucial scaffolds for the developpement of surfactants, preservatives or drugs. Despite the presence of the acidic function, they could be rather insoluble in aqueous media. Their solubility could nevertheless be increased through the esterification of the anomeric position of glucose or glucuronic acid. [1] This acylation step is an essential process in the detoxification of many drugs *in vivo* and is also critical in the field of food additives or surfactants. Glycosyl esters were mainly obtained by chemical coupling and only rare examples of enzymatic processes has been described in the litterature. They mostly relied on glycosyl transferases obtained from microsomial preparation. Indeed the diversion of glycosyl hydrolase to perform such anomeric acylation is hampered by the poor nucleophilicity of carboxylic acid and the strong competition with water. In order to switch off the water activity, we decided to convert a glycosyl hydrolase, an α-L-arabinofuranosidase GH51 from *Clostridium thermocellum* (*Ct*Araf51) into thioligase by mutation of the acid-base residue and evaluate its potential as an acyl transfer catalyst.

*Ct*Araf51 was already known as a versatile catalyst in both tranglycosylation and thioligation reactions to obtain biologicaly relevant arabino and galactofuranoconjugates.[2,3] We report herein a new catalytic activity for this enzyme, the *C*-1 acylation in the presence of an activated arabinofuranose donor and a carboxylic acid acceptor.[4] In spite of the weak nucleophilicity of the carbonylated acids, this new activity for a GH could be exemplified and very good yields (18% to 83%) could be obtained depending of the nature of carboxylic acid (Figure 1). This methodology shows the plasticity of glycosylhydrolases and in particular the arabinofuranosidase towards numerous catalytic activities.

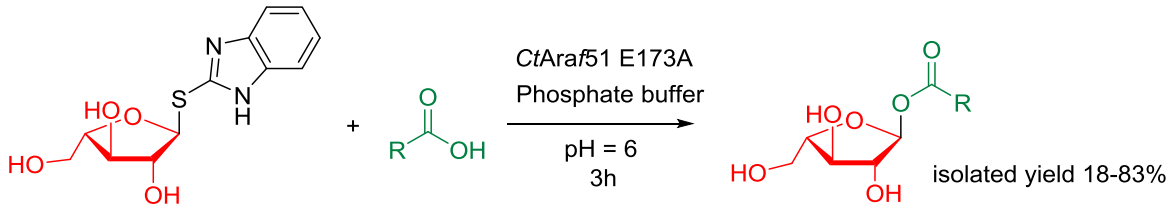

Figure 1. New C_1-acylation activity of *Ct*Araf51.

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Chemoenzymatic Synthesis of Tumor-Associated Carbohydrate Antigen Disialogalactosylgloboside (DSGG)

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Sialylation in glycan is important in physiological system. There are a lot of bacterial sialytransferase has been report, but the regioselectivity of these STs has rarely been discussed. Using the globo-series glycans as the substrates, we found the regioselectivity of Pd2,6ST and Psp2,6ST got major difference, and this difference benefited us to synthesize some unique glycans with excellent yield.

For example, utilizing one-pot multienzyme (OPME) sialylation system, we found that the sialylation of Gb4 (GalNAcβ1-3Galα1-4Galβ1-4Glc) occurred only at terminal 4GalNAc. When Gb5 (Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glc) was used as an acceptor, surprisingly, both of this two STs prefer the terminal 5Gal. This may be due to the more stereo-hindrance at 4GalNAc of Gb5 which close to the 3Gal with α-linkage. Based on this result and literature report, the DSGb5 (Siaα2-3/Siaα2-6Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glc) can be synthesized with high specificity by using SSEA4 (Siaα2-3Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glc) as a substrate under the sialylation of Pd2,6ST. However, the DSGG (Siaα2-3Galβ1-3/Siaα2-6GalNAcβ1-3Galα1-4Galβ1-4Glc) was synthesized under the Psp2,6ST treatment using the same SSEA4 substrate. This result suggest α2-3-sialylation at terminal Gal can increase the sialylation of Pd2,6ST at the 6-OH of terminal Gal, and may decrease the sialylation of Psp2,6ST at the 6-OH of terminal Gal. Compare to Lactose, the reaction rate of Psp2,6ST was apparently decreased when Sialyl Lactose was used.

In conclusion, we have found the regioselectivity of Pd2,6ST and Psp2,6ST in this study, and the information will help us to synthesize the related glycans with high yield.

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Identification of a pivotal residue for determining the block structure from alginate C-5 epimerases

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Keywords: alginate, epimerases, protein design

Alginate is a linear co-polymer composed of (1-4) linked β-D-mannuronic acid (M) and its epimer α-L-guluronic acid (G). The polysaccharide is first produced as homopolymeric mannuronan and only at the polymer level M residues are converted to Gs by epimerases. The bacterium *Azotobacter vinelandii* encodes a family of seven secreted and calcium ion-dependent mannuronan C-5 epimerases (algE1-algE7) [1-3] that consist of two types of structural modules; the A-modules which contain the catalytic site of the enzyme and the R-modules involved in substrate and calcium binding, increasing the activity of the enzyme. In this study, we made rational design of new hybrid mannuronan C-5 epimerases constituted by the A-module from AlgE6 and the R-module from AlgE4, based on the primary and tertiary structure analysis. This approach led to a better understanding of the molecular mechanism being determinant of the different product profile for MG-block or GG-block forming enzymes. A long loop with Tyr or Phe extruding from the β-helix of the enzyme has proven essential in defining the final alginate product, and its involvement is likely to be associated with substrate binding.

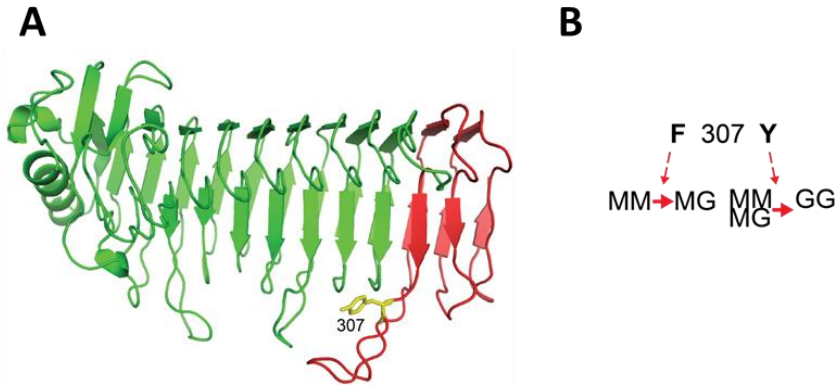

Figure 1. (A) Ribbon structure of AlgE6 A-module represented with green (belonging to AlgE6) and red (belonging to AlgE4) colors as in point mutant AlgE64_BF307Y. In yellow Tyr307 which is the point mutation of AlgE64_BF307Y. Tyr307 belongs to a loop in AlgE6 structure in proximity of the substrate binding groove. The model structure was obtained using SWISS-MODEL database [4]. (B) It is hypothesized that residue 307 being phenylalanine the epimerases forms alternating (MG) block structure while being a Tyrosine will result in both MG and GG-block formation.

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Applications of Galactose Oxidase in Carbohydrate Chemistry

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Keywords: Galactose Oxidase, oxo-glycosides .

Selective chemical oxidation of carbohydrates is very challenging. Galactose oxidase (GOase), a copper dependent oxidase, can oxidize the primary hydroxyl of non-reducing hexoses with strict regiospecificity and can be carried out in buffer whilst requiring only ambient conditions and oxygen as a co-substrate. Two variant GOases have now been used to oxidize of panel of glycosides. The industrial potential of GOase was tested with the gram scale oxidation of lactose (an inexpensive renewable feedstock) that was achieved to full conversion in batch. It was evident that oxygen availability is crucial for the reaction to proceed on gram scale and was the limiting factor in scale ups. To overcome the issue of oxygen availability, a continuous flow system was utilized for the bio-oxidation of lactose yielding multi gram quantities of a high value 6-oxolactose. Oxidation of industrially relevant glucosides was achieved with GOase F_2; chain length and linkage type had an impact on conversion. The broad substrate scope of GOase F_2 and the scalability of M_1 show the enzyme to be a highly useful tool in carbohydrate chemistry for selective functionalization of the primary hydroxyl.

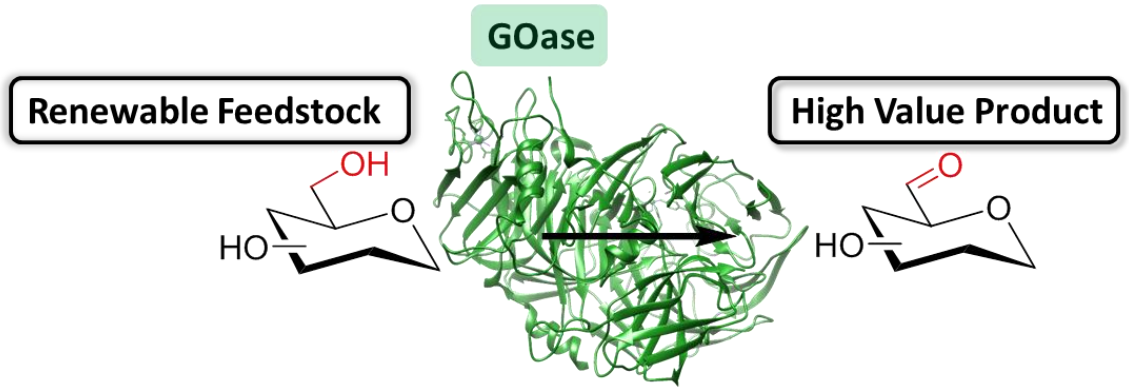

Figure 1. Generation of 6-oxo glycosides through oxidation with Galactose Oxidase

Fructosylation of phenolic compounds by levansucrase from *Gluconacetobacter diazotrophicus*

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Keywords: Levansucrase, fructosylation, phenolic compounds.

Phenolic compounds constitute an important source of bioactive molecules. They have some health benefits such as anti-inflammatory, antiviral, anti-tumor and anti-oxidant activities, however for most of them the bioavailability is a problem due to their hydrophobic nature [1]. Thus, enzymatic glycosylation of phenolic compounds is getting importance to increase the solubility, stability and bioavailability [2]. Levansucrase (LsdA) from *Gluconacetobacter diazotrophicus* (EC 2.4.1.10) is a fructansucrase from the Glycoside Hydrolase (GH) family 68 that catalyzes the de novo synthesis of fructooligosaccharides from sucrose, a readily available and cheap agroressource. This bacterial enzyme may also fructosylate exogenous hydroxylated acceptor molecules, with efficiencies that depend on the acceptor recognition [3]. In this work, levansucrase from *G. diazotrophicus* was evaluated for the fructosylation in aqueous solution of different phenolic compounds. The reaction was positive for puerarin (93 %), coniferyl alcohol (25.1 %), rosmarinic acid (15.2 %) and catechin (10.9 %). The synthesis of di, three and tetra fructosides was observed for some phenolic compounds. When DMSO was used as co-solvent the conversion rate increased for mangiferin (13 %) and resveratrol (5 %). In the case of the principal fructoside produced (puerarin monofructoside), the structure was determined by NMR and the compound present a fructosyl moiety linked to the 6-position of the glucosyl moiety of puerarin. Regarding the production of new fructosides from phenolic compounds, it is the first time that enzymatic fructosylation of ferulic acid, caffeic acid, rosmarinic acid, coniferyl alcohol and resveratrol was performed. Due to its high activity and wide acceptor promiscuity, *G. diazotrophicus* levansucrase is a promising candidate for biotech application in the fructosylation of different phenolic compounds.

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Hen Egg-White Lysozyme Engineering for the synthesis of chitinoligosaccharides

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Keywords: Enzyme engineering, Chemoenzymatic synthesis, Chitinoligosaccharides, Glycosynthase.

Chitinoligosaccharides (COs), β-1,4-linked oligomers of *N*-acetylglucosamine, are an important class of signaling molecules involved in plant-biosphere cell-cell interactions. COs having a degree of polymerisation (DP) from 6 to 8 are potent inducers of immune responses in rice and wheat among others [1]. Despite their biological interest and potential agronomical usefulness, COs with well-defined structure remain poorly accessible. While chemical or enzymatic degradation of chitin allows the production of COs of DP 2 to 6, higher oligomers are hardly accessible by depolymerisation methodologies. Therefore, enzymatic synthesis of COs has been a matter of research by exploiting the transglycosylation activity of retaining glycoside hydrolases (GH).

In the present work, we report the engineering and expression of Hen Egg-White Lysozyme (HEWL, GH-22) in the methylotrophic yeast *Pichia pastoris*. Site-directed mutagenesis on the essential aspartate 52 [2] was carried out, three mutants devoid of hydrolytic activity were produced and one of them (D52S) displayed an efficient glycosynthase activity. Polycondensation reactions of α-chitintriosyl fluoride led to the formation of COs up to DP 15. Afterwards, we took advantage that a de-*N*-acetylated oligomer at the non-reducing end cannot behave as an acceptor for HEWL (+1 subsite does not accept a glucosaminyl residue). In a one-pot sequential procedure, the donor was first specifically de-*N*-acetylated at the non-reducing end by the action of Nod B chitin deacetylase, and then condensed on COs acceptors with mutant HEWL to give single addition products with size varying from hexa- to octamer.

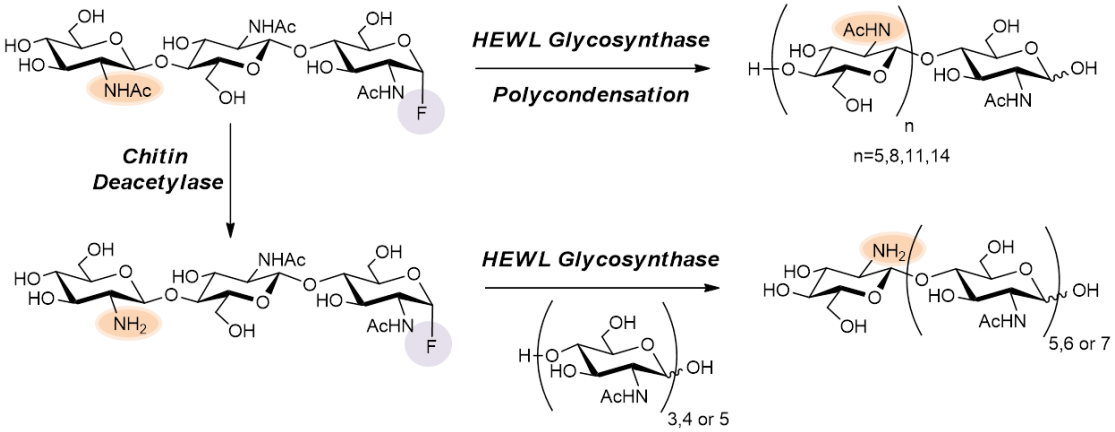

Figure 1. Synthesis of long chitinoligosaccharides by HEWL glycosynthase.

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Improvement of cell surface expression of β-glucosidase on yeast by overexpression of GPI biosynthesis proteins

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Keywords: β-glucosidase, cellulase, *Saccharomyces cerevisiae*, cell surface display

Heterologous expression of cellulolytic enzymes on cell surface of *Saccharomyces cerevisiae* is one of promising strategy for development of a microbial strain suitable for consolidated bioprocess (CBP) from cellulosic biomasses [1]. However, an important limitation rely on this technology is that the expression level of cellulolytic enzymes on yeast cell surface is not enough to complete hydrolysis of biomass. As an attempt to overcome this obstacle, various cellulolytic enzymes (cellobiohydrolase, endoglucanase, and β-glucosidase) were screened in order to identify a suitable enzymes to be immobilized on *S. cerevisiae* cells. Then, with the purpose of enhancing cellulase expression level on the cell surface, a number of native genes involved in glycosylphosphatidylinositol (GPI)-anchored protein biosynthesis were constitutively expressed in the β-glucosidase-displaying *S. cerevisiae* strain. Effect of overexpression of GPI biosynthesis proteins on β-glucosidase activity was evaluated by comparing with the transformant transformed with empty vector. We found that the β-glucosidase activities on the cell surface of yeast transformants containing additional copy of GPI biosynthesis genes were varied depend on type of GPI biosynthesis genes. The maximum β-glucosidase activity was obtained with the LAS21-overexpressed transformant, with approximately 49% higher activity compared to the control strain. This work suggests the potential engineering approach for the improvement of cellulase-displaying yeasts that will be useful for the cost-effective CBP development.

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Facial synthesis of Globo H analogs conjugated vaccines and their immunogenicity studies

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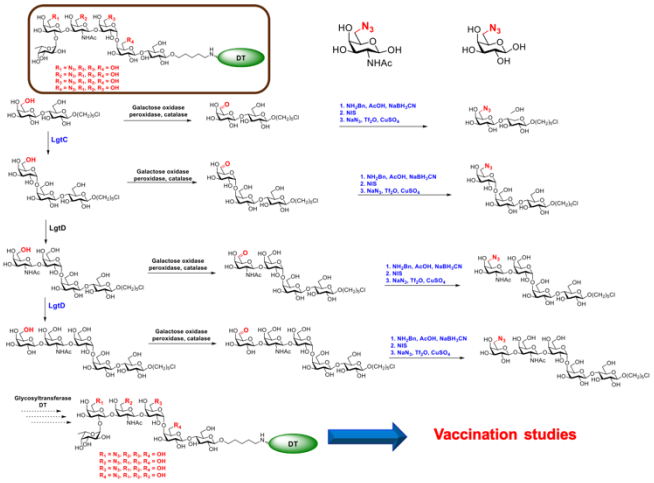
Keywords: Globo H, Galactose oxidase, Vaccine.

Globo H is a potential cancer vaccine due to it is overexpressed on many types of cancer cell but rarely expressed on normal tissue. Globo H based cancer vaccines have been used in the clinical trials including breast, ovarian and prostate cancers, but it is still to be improved due to the low immunogenicity. Previous reports indicated that non-self antigens modified carbohydrate-based vaccines induce stronger immunogenicity. However, the relationship between site specific modification and immunogenicity has not been elucidated. To elicit stronger immunogenicity of Globo H conjugated vaccines, we synthesized different Globo H analogs with non-self antigens modifications. The modifications were installed at the C'6 of Gal or/and GalNAc on different moiety of Globo H by chemoenzymatic methods. We adopted galactose oxidase which showed excellent regio-selectivity to oxidize the non-reducing end galactose of lactose, GB3, GB4, and GB5. The terminal C'6 oxidized oligosaccharides can be converted into different derivatives in few steps and elongated to Globo H analogs by glycosyltransferases in ideal yeild. These Globo H analogs were conjugated with carrier protein CRM^{197} (DT) as the vaccine candidates for immunization studies. In this study, we have synthesized series of Globo H analogs conjugated vaccines in efficient steps. In the future, we will utilize the glycan array to examine the antibody titer from the immunized mice and explore which modification or modified position can provide strong immunogenicity for future vaccine development.



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A novel family of LPMO acting on cellulose identified in the secretomes of *Aspergillus* spp.

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Keywords: Filamentous fungi, plant biomass, secretomes, LPMO

Lignocellulosic biomass is considered as a promising alternative to fossil resources for the production of fuels, materials and chemicals. In order to use this abundant feedstock, efficient enzymatic systems are needed to degrade the plant cell wall and overcome its recalcitrance. A widely-used producer of hydrolytic cocktails is the ascomycete *Trichoderma reesei*, which is known for its high cellulases secretion and benefits from decades of strain improvement; however, this organism secretes only a limited set of enzymes [1]. To compensate this lack of diversity, which may be a bottleneck for saccharification yields improvement, one strategy is to upgrade the *T. reesei* enzyme cocktail with enzymes produced by other biomass-degrading filamentous fungi isolated from biodiversity [2].

In order to look for such enzymes, five strains from the genus *Aspergillus* were grown on several inducers to produce various secretomes. These enzymatic cocktails were tested for their ability to boost a *T. reesei* reference cocktail for the saccharification of pretreated biomass. Proteomic analyses of fungal secretomes that significantly improved the degradation of biomass showed the presence of proteins belonging to a new family of Lytic Polysaccharides Monooxygenases (LPMOs), a recently discovered class of oxidative enzymes that raised a high interest due to their ability to boost the degradation of lignocellulosic biomass [3]. Members of this novel LPMO family are encountered in fungi and oomycetes with life styles oriented toward interactions with plant biomass. Several member of the family were recombinantly produced for further characterization. Oxidative degradation of cellulose was demonstrated, and one of the produced proteins was found to significantly improve the activity of *T. reesei* cellobiohydrolase (CBHI) on cellulosic substrates, which could be of great interest for use in biorefineries.

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Reinvestigating light-driven LPMO reactions and the role of reactive oxygen species

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Keywords: LPMO, chlorophyllin, titanium dioxide, ROS.

In 2016, two studies showed that light may be used to fuel LPMO activity on cellulose. By exposing a combination of pigments (e.g chlorophyllin, Chl) and a reductant to light, Cannella et al. [1] achieved a two-orders of magnitude boost in LPMO activity. In another study, Bissaro et al. [2] showed that vanadium-doped titanium dioxide (V-TiO_2) exposed to visible light can fuel LPMOs. At the time, molecular oxygen (O_2) was considered to be the *only* co-substrate of LPMOs during oxidative cleavage of glycosidic bonds. However, later in 2016 [3], it was demonstrated that LPMOs may use H_2O_2 as co-substrate, carrying out a peroxygenase reaction, which is much more efficient than the *apparent* monooxygenase reaction [4].

We have revisited these studies from 2016 and investigated whether reactive oxygen species, in particular hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\bullet-}$), play a role in light-driven LPMO activity under standard aerobic conditions. We used coupled enzymatic assays to probe the roles of $O_2^{\bullet-}$ and H_2O_2 in the oxidation of Avicel by AA10C from *Streptomyces coelicolor* (*Sc*AA10C) when fueled by light-exposed V-TiO_2 or by the Chl/light system. The results demonstrate the feasibility of light-driven peroxygenation of polysaccharides and suggests that the LPMO activity observed in light-driven systems may be due to the controlled generation of H_2O_2 in such systems.

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Discovery and characterization of an atypical fungal lytic polysaccharide monooxygenase (LPMO) family

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Keywords: LPMOs, cellulose, crystallography, immunolabeling.

Since 2010, six different families of lytic polysaccharide monooxygenases (LPMOs) have been identified and characterized (AA9-AA11/AA13-AA15) [1, 2, 3, 4, 5, 6]. They are found in a wide range of organisms such as fungi, insects, bacteria to act on recalcitrant polysaccharides. Known LPMO families differ in terms of substrate specificity but the two active site histidine residues are strictly conserved to form the histidine-brace that coordinates the copper ion. Using post-genomic approaches, we identified a new LPMO family in the secretomes of fungal saprotrophs. All members of this new fungal LPMO family harbor a C-terminal glycosylphosphatidylinositol (GPI) anchor. Four members originating from various fungal lineages were recombinantly produced and showed activity on cellulose with C1 regioselectivity. Resolution of the three-dimensional structure revealed a reduced substrate binding surface (compared to that commonly encountered for other LPMOs) which displays unusual copper ligands in addition to the histidine brace. In the case of the ectomycorrhizal symbiont *Laccaria bicolor*, immunolabelling experiments showed that the LPMO was specifically produced at the interface between fungal hyphae and *Populus* rootlets (ectomycorrhizae), suggesting that this LPMO acts as an enzymatic effector of plant cell wall remodeling to promote the *Laccaria-Populus* symbiosis. The discovery of this atypical LPMO family offers new prospects related to the biological role of LPMOs *in vivo*.

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Understanding enzyme-substrate interactions in Carbohydrate Esterase Family 15

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Keywords: CE15, glucuronoyl esterase, glucuronoxylan, lignin-carbohydrate complexes.

Carbohydrate Esterase Family 15 (CE15) is a rather small family, comprising approximately 200 members, which was established in CAZy (www.cazy.org) in 2006. The family was created following the characterization of a glucuronoyl esterase (GE) from the fungus *Schizophyllum commune* [1], which was shown to cleave methyl moieties ester-linked to the O6 position of glucuronic acid. CE15 enzymes are proposed to cleave ester linkages between lignin and glucuronoxylan, so-called lignin-carbohydrate complexes (LCCs), which are important features in biomass recalcitrance. We recently characterized ten new GEs from three bacterial species and solved the structures of two of these, essentially doubling both the biochemical and structural data available for the family [2].

An in-depth understanding of how CE15 enzymes interact with their complex substrates is still lacking, as only one structure with a monosaccharide ligand has been solved to date [3]. To address this, we have pursued solving new GE structures and obtaining protein-ligand complex structures. The studies have resulted in a novel structure exhibiting features with prominent inserts surrounding the active site, suggesting different specificities between bacterial and fungal GEs. In addition, we have solved the first structures of a CE15 enzyme with larger ligands, which gives direct evidence of how these enzymes interact with the different parts of its proposed physiological LCC substrates. Combined with kinetic characterizations, these new investigations greatly add to the knowledge of enzyme-substrate interactions in CE15 and enhances how these enzymes may act in natural conditions, which could aid in industrial biomass conversion.

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Pyranose oxidase from the Actinobacterium *Kitasatospora aureofaciens*: a role in redox cycling?

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Keywords: pyranose oxidase, manganese peroxidase, Actinobacteria, lignin degradation.

We present a comprehensive biochemical and phylogenetic characterization of a novel pyranose 2-oxidase from the actinomycetous bacterium *Kitasatospora aureofaciens* (*Ka*POx) as well as a potential synergism with peroxidases in lignin metabolism. Pyranose 2-oxidase (POx) has long been accredited a role in lignin degradation, but insight into the mechanisms and interactions is insufficient. There is ample data in literature on the oxidase and dehydrogenase activities of POx [1, 2], but a biological relevance of this property could not be established conclusively.

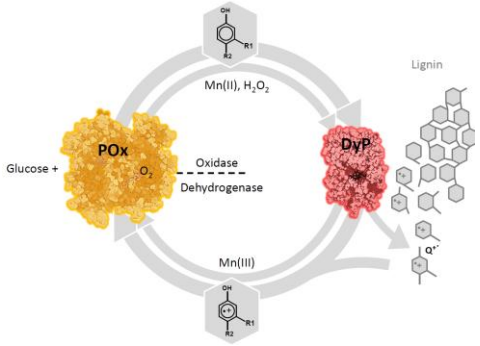

Figure 1. POx utilizes monosaccharides to supply H_2O_2 to peroxidases (DyP). Aromatic radicals and Mn(III) complexes produced by the peroxidase can be re-reduced by POx, preventing repolymerization and cellular damage.

A phylogenetic analysis of fungal and bacterial (putative) POx-encoding sequences revealed their close evolutionary relation and supports a late horizontal gene transfer of ancestral POx sequences into fungi. We successfully expressed and characterized a novel bacterial POx from *K. aureofaciens*, one of the genes closely related to well-known fungal POx. Its biochemical characteristics comply with most of the hallmarks of known pyranose 2-oxidases. We further performed redox cycling of aromatic lignin model compounds between *Ka*POx and manganese peroxidase (MnP). In addition, we found a Mn(III) reduction activity in *Ka*POx which, in combination with its ability to provide H_2O_2, implies this and potentially other POx as complementary enzymes for oxidative lignin degradation by specialized peroxidases.

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A Kinetic Study of a Single Lytic Polysaccharide Monooxygenase on Different Types of Substrate

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Keywords: LPMO, Kinetics

The efficient conversion of chitin and cellulose biomass into easily accessible sugars is important in a sustainable manufacture of products of value. A key advancement in the enzymatical breakdown of these tough materials are the discovery of lytic polysaccharide monooxygenases (LPMOs) in 2010. The oxidation reaction catalyzed by LPMOs opens the surface of polymeric sugars and enables the attack of hydrolases resulting in a more efficient saccharification. Since LPMOs are a rather young class of enzymes and not fully understood yet, it is important to know their kinetic properties. Kinetic data will not only help to design enzyme catalyzed reactions in the most efficient way, they will also help to understand the biological role of the enzyme, show limitations for potential applications and reveal possible targets for genetic engineering. Here, we present detailed kinetics on an LPMO catalyzed reaction on different substrates using two different methods. The obtained data increases our understanding of the mechanistic action of these remarkable enzymes.

Novel two-domain bifunctional acetyl esterase/endo-xylanase for Xylooligosaccharides production

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Keywords: Carbohydrate esterase, Endo-xylanase, Xylan, Xylooligosaccharides.

A novel bifunctional esterase/xylanase from *Clostridium "boliviense"* strain E-1 (*E1*XynES) was recombinantly produced in *E. coli* Rosetta-Gami(DE3). The esterase/endo-xylanase has a total molecular weight of 44,2 kDa, divided in two-domains, one Carbohydrate Esterase (family CE1) and one Glycoside Hydrolyse (family GH11) (Figure 1). The kinetics constants for both activities were determined using *p*-Nitrophenyl acetate (*p*NPAc) and *p*-Nitrophenyl xylobioside (*p*NPX$_2$) as substrates, V_{max} (μmol·min^{-1}) and K_m (mM) showed values of 0,003 and 0,233 for *p*NPX$_2$ and, 0,011 and 2,25 for *p*NPAc, respectively. *E1*XynES carbohydrate esterase domain was active using not only in *p*NPAc, but also using *p*-Nitrophenyl butyrate. On the other hand, although sequence comparison showed close relation with feruloyl esterase, *E1*XynES did not show detectable activity using *p*-Nitrophenyl trans-ferulate. Xylanase activity was measured using *p*NPX$_2$, determining endo-xylanase activity. Xylosidase activity however, was not observed when *E1*XynES was tested over *p*-Nitrophenyl xylopyranose (*p*NPX) as substrate. The activity profile determined *E1*XynES attractive for production of Xylooligosaccharides (XOs). Then, *E1*XynES was tested over Birchwood xylan, debranched Wheat arabinoxylan and Quinoa stalks glucuronoarabinoxylan. Products profile analyzed by HPAEC-PAD showed production of xylotriose and xylotetraose as major hydrolysis products. Interestingly, the pattern of XOs production was different to the produced by xylanases from families GH10 and GH11, commonly used. *E1*XynES is determined highly suitable to XOs production, avoiding monosaccharide (xylose) production. Furthermore, carbohydrate esterase bifunctionality is of interest as an "accessory" activity to the xylanase, specifically for its primary function determined by enzyme kinetics.

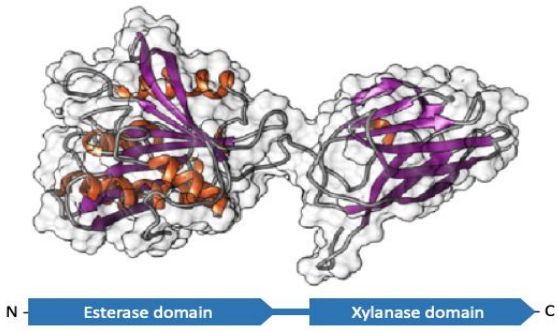

Figure 1. Computational model of bifunctional esterase/xylanase (*E1*XynES). Esterase domain CE1 was modelling from feruloyl esterase of *C. thermocellum* and xylanase domain GH11 was modelling from endoxylanase of *C. cellulolyticum.*

Mass spectrometry as a sensitive tool to study specificity of carbohydrate oxidizing enzymes

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Keywords: carbohydrate, oxidase, dehydrogenase, AA3, AA5, mass spectrometry

Oxidative enzymes targeting carbohydrate substrates include carbohydrate oxidoreductases and monooxygenases that are presently classified into various auxiliary activity (AA) families within the CAZy database. Due to multiple hydroxyl groups to be potentially oxidized in mono-, oligo- and polysaccharides, the analysis of products is not straightforward. Oxidation of the anomeric carbon C1 is resulting in lactone which spontaneously hydrolyses to carboxylic acid in water. Oxidation of primary hydroxyl, for example in C6 of hexoses, results in an aldehyde, which may be further oxidized to carboxylic acid. Secondary hydroxyls are oxidized to ketones. Due to reactivity of carbonyl groups (aldehydes, ketones) they exist primary as hydrates (germinal diols) in water, which is further complicating the analysis.

Mass spectrometry (MS) is a sensitive and fast analysis method which can be coupled to gas or liquid chromatography. Samples can be also applied by direct injection after desalting. We have developed a reliable GC-MS method to quantify the degree of oxidation of oligo- and polysaccharides by AA5 family enzymes, such as galactose oxidase. The method is based on labeling the oxidized galactosyl residue by reduction with $NaBD_4$. MS determination of labeled, deuterated galactose after acid methanolysis was used to quantify the degree of oxidation [1]. In the recent studies the $NaBD_4$ reduction was applied to xylooligosaccharide samples oxidized by AA3 family pyranose dehydrogenases. The samples were analyzed by the Quadrupole Time-of-flight MS in the negative mode, which has proven to be better suited for complex oligosaccharide structural analysis than the typically used positive mode [2]. By following the labeled, deuterated residues, the mono- and dioxidized residues in oligosaccharides up to DP6 could be identified and quantified.

We have also used combination of isotopic labeling and negative-mode electrospray ionization-ion trap MS/MS to proof the further oxidation of formed aldehyde to a carboxylic acid by AA5 family enzymes, the reaction which has been controversial [3]. Inherent property of carbonyl groups to form hemiacetal conjugates with alcohols, like methanol, instead of water (hydrate formation), was proven to result in a feasible diagnostic adduct to identify the oxidized residue applying MS/MS analysis [3]. The present paper will discuss different MS-based methods in combination with labeling techniques to study efficiency and specificity of carbohydrate oxidizing enzymes.

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Glycosaminoglycan mimetics from marine bacterial exopolysaccharide and innovative concepts for their structural elucidation

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Keywords: Marine bacteria, exopolysaccharides, low molecular weight derivatives, chemical structure, function.

In research of new bioactive molecules, bacteria from marine origin constitute a considerable source of innovative molecules. In particular, exopolysaccharides (EPS) produced by these bacteria become a renewable source of biocompatible and biodegradable molecules. The GY785 EPS (Figure 1), produced by the deep-sea hydrothermal vent strain *Alteromonas infernus*, is an anionic branched high molecular weight heteropolysaccharide with a nonasaccharide repeating unit. The low molecular weight derivatives of this EPS have previously shown to display some interesting GAG-like properties [1, 2].

However, two questions remain unanswered: what is the accurate structure of the obtained derivatives? And how do the modifications affect the biological properties of the derivatives?

To answer to these questions, oligosaccharide fragments that are a constitutive part of the GY785 EPS will be obtained by either enzymatic degradation [3] or organic synthesis. These derivatives provide innovative tools to elucidate both chemical structure of complex EPS and structure-function relationship.

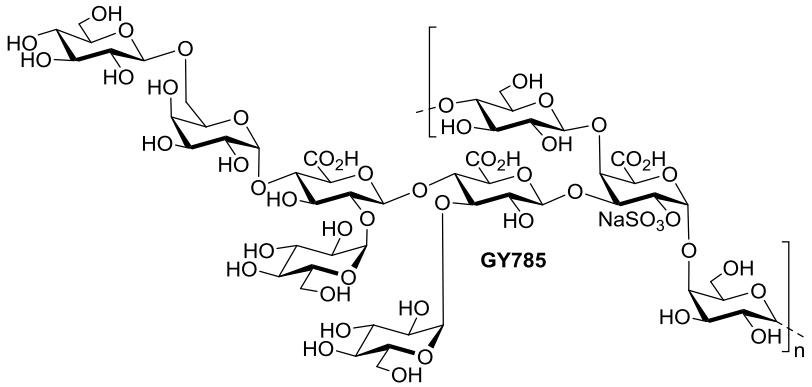

Figure 1. Repeating unit of GY785 exopolysaccharide produced by *Alteromonas infernus* bacterium.

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Enzymatic synthesis of functionalised β-mannosyl conjugates from renewable hemicellulosic glycans

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Keywords: Enzymatic synthesis, β-mannanase, Glycosyl-acrylates, Hemicellulose.

Major research efforts are currently directed toward finding sustainable alternatives to fossil resources in the fields of energy, chemical and material production. The expanding field of plant biomass utilisation and valorisation is thus highly desirable from several perspectives.

In contrast to a chemical approach, enzymatic activity generally occurs in low temperatures and with no or little need for other chemicals than the reactants, thus resulting in greener synthesis pathways. Fractionating and refining of waste streams from the forestry industry enables well defined reaction conditions and plant glycans for further enzymatic processing [1].

We have recently shown the ability of family 5 β-mannanases to *via* transglycosylation, catalyse the formation of glycosidic bonds between the hemicellulosic glycosyl donor, AcGGM (Acetyl-Galactoglucomannan), and functional acceptors 2-HEMA (2-hydroxyethyl methacrylate) and allyl alcohol (2-propen-1-ol), the products' subsequent LC purification, as well as, NMR structure determination and radical polymerisation of the novel mannosyl-acrylates [2].

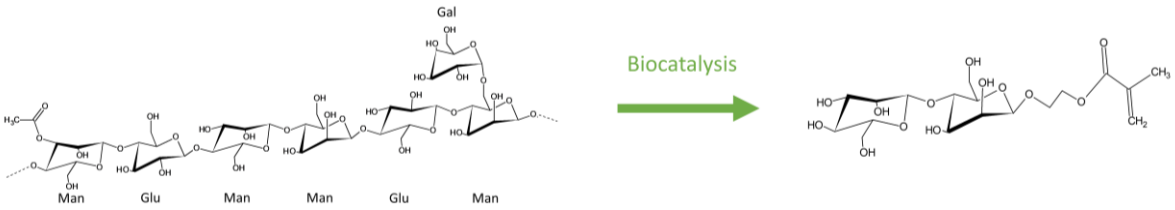

Figure 1. Schematic representation of target glycosyl substrate, AcGGM, and product, β-mannobiosyloxyethyl methacrylate from *Tr*Man5A biocatalysis.

By altering the composition of such functionalised glycoconjugate monomers in biopolymers, it is expected that material properties can be finetuned for the desired application. Furthermore, rational design and selection of the enzyme catalyst alters product profiles and should be used as a tool to minimise the need for downstream processing of these novel biomaterials.

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Enzymatic degradation of the polysaccharide extracellular matrix of the microalga *Botryococcus braunii*

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Keywords: enzyme, polysaccharide, extracellular matrix, degradation.

Microalgae are photosynthetic microorganisms capable of producing a range of interesting biochemicals such as pigments, vitamins or hydrocarbons. Their high growth rate makes them more interesting than higher plants for biomass production. However these high value molecules are rarely released in the culture medium and it is generally difficult to extract them because of the cell wall and the presence of an extracellular matrix. Mechanical or chemical processes destroy the cells and sometimes alter the molecules.

The microalga *Botryococcus braunii* produces hydrocarbons that can represent more than 40% of their dry weight and can be converted into fuels [1]. The advantage of using *B. braunii* resides in the fact these hydrocarbons are excreted [2]. Unfortunately they are retained in the extracellular matrix partly composed of a polysaccharide sheath containing mainly arabinose and galactose, whereas the cell wall is composed of β-1,3 and/or β -1,4 glucans [2].

Our goal is to use glycosidases to destabilize the polysaccharide extracellular matrix in order to release the hydrocarbons they produce and to extract them by keeping the cells viable. An endoglycanase from the GH9 family and an arabinanase from the GH43 family were found to be active on this microalga. Flow cytometry was used to follow the evolution of the size of the colonies in the presence of these enzymes. The efficiency of the endoglycanase was increased by fusion with a CBM3.

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Deep-sea hydrothermal vent bacteria: inexhaustible source of glycosaminoglycan-mimetic exopolysaccharides

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Keywords: exopolysaccharide, glycosaminoglycan, uronic acid, hexosamine.

Bacteria have developed a unique strategy to survive in extreme adverse conditions through a synthesis of an extracellular polymeric matrix conferring to the cells a protecting microenvironment. The important structural component of this complex network constitutes high-molecular weight hydrophilic macromolecules, namely exopolysaccharides (EPS). EPS composition with the presence of particular chemical features may closely be related to the specific conditions in which bacteria evolve. Deep-sea hydrothermal vent bacteria have already been shown to produce EPS rich in hexosamines and uronic acids, frequently substituted by sulfate groups. Such a particular composition ensures interesting functional properties, including biological activities mimicking those known for glycosaminoglycans (GAG).

The aim of the present study was to go further into the exploration of the deep-sea hydrothermal vent collection of bacteria to discover new strains able to excrete EPS endowed with GAG-like composition. After screening of the whole collection containing 692 strains, 38 bacteria have been selected for the EPS production at laboratory scale. Chemical characterization of the EPS evidenced their high chemical diversity with the presence of atypical compositional patterns. These EPS constitute potential bioactives for a number of biomedical applications, amongst regenerative medicine and cancer treatment.

Metabolic engineering strategies for glycolipids production

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Keywords: glycolipids, metabolic engineering, glycosyltransferase, phosphatidic acid.

The synthesis of carbohydrates and glycoconjugates has been attracted a lot of attention for many years because of their relevant biological roles and their diverse applications in biomedicine, biomaterials and food fields. Nowadays chemistry and biotechnology aim to afford these compounds with the precise regio- and stereoselectivity and with high productivity [1,2].

Glycolipids are of special interest as immunostimulants and drug delivery systems [3,4]. The understanding of the glycosyltransferase MG517 as drug target to prevent Mycoplasma infections has driven this enzyme as a potential biocatalyst for production of glycolipids [5,6]. *E. coli* does not present glycolipids and MG517 was shown to be functional catalysing the reaction between UDP-Glc and diacylglycerol, common metabolites in *E. coli*, and producing glycolipids as new compounds [7]. They are able to accumulate in the membrane and modulate membrane composition. We report different strategies to increase on the one hand, the availability of fatty acid and to modulate phosphatidic acid, precursor of diacylglycerol and phospholipids intermediate, and on the other hand, UDP-Glc to impact on glycolipid production.

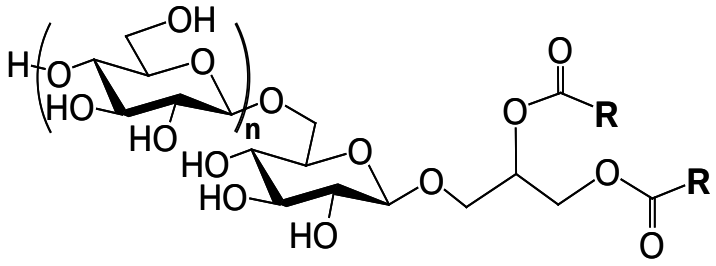

Figure 1. Glycoglycerolipids structure to produce in *E. coli*. R corresponds to the main fatty acids present in *E. coli*

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IBISBA 1.0 provides the infrastructure framework for translational research services in Industrial Biotechnology

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Keywords: Industrial Biotechnology, Synthetic Biology, Bioeconomy, Circular Economy.

Industrial Biotechnology, so-called Key Enabling Technology of the bioeconomy, is at a crucial point in its development, because it has been increasingly empowered by progress in life sciences research, and is now benefitting from synthetic biology.

To move industrial biotechnology further along the road to industrial maturity, progress in Research & Innovation is required to better translate new knowledge into innovative pre-industrial processes that can be taken up by industry. This is vital to support the development of the European industrial biotechnology sector and Europe's circular bioeconomy transition.

Europe possesses a lot of research infrastructures that can be used to accelerate the development of innovative bioprocesses. Currently, these are mostly disconnected and thus unable to host the development of efficient R&I project pipelines. However, networking of individual infrastructure facilities is a viable way to overcome this problem.

IBISBA 1.0 (www.ibisba.eu) [1] is a research infrastructure project aiming to create and operate a Pan-European coordinated research infrastructure network to provide innovation services to industrial biotechnology. These services include the hosting of bioprocess development projects, helping to translate results into preindustrial innovation, the development of experimental workflows and standards to improve interoperability and reproducibility, a web-based repository for knowledge asset management and first-rate training for early career stage researchers. As a step towards reaching these ambitions, IBISBA 1.0 is operating a transnational access (TNA) programme, which provides subsidized access to a set of research facilities. The TNA programme is open to all eligible researchers wishing to translate their research results into pre-industrial innovation. In this framework, the Italian ProtEnz-IBBR installation provides services for the identification and characterization of novel CAZymes from extremophiles to be exploited for biotechnological applications.

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[1] Industrial Biotechnology Innovation and Synthetic Biology Accelerator (IBISBA) 1.0 - H2020-INFRAIA Grant agreement n. 730976. Contact info: Michael O'Donohue, Project Coordinator. michael.odonohue@insa-toulouse.fr

Design of a novel chimeric dextransucrase fused to the carbohydrate binding module CBM2a

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Key words: dextransucrases, cellulose, dextrans, *Leuconostoc*, affinity purification, lignocellulosic

The lactic acid bacteria (LAB) have great potential to produce homoexopolysaccharides (hEPS), which have been the subject of extensive research efforts, given their health benefits and physicochemical properties. The hEPS are a group of polymers whose functional properties are determined by structural characteristics of varied molecular weights, types of glycosidic linkages, degrees of branching and chemical composition. The dextransucrase enzymes (DSases) are responsible of the synthesis of a kind of hEPS (dextrans), which are among the first biopolymers produced at industrial scale with applications in medicine and biotechnology. The concept of glycodiversification opens up even wider the application field of DSases. In that sense the isolation and characterization of new DSases is of prime importance. We describe the isolation and characterization of a novel extracellular dextransucrase (EC 2.4.1.5) encoding gene. An amplicon of 4659 pb (obtained by Long and Accurate PCR) codes for the protein DSR-F of 170 kDa with a signal peptide of 38 amino acids. The comparison of the deduced amino acids sequence with other DSases deposited in the GenBank show the DSR-F belongs to the GH70 family. From DSR-F we obtain an active truncated variant (DSR-F-ΔSP-ΔGBD), and DSR-F-ΔSP-ΔGBD-CBM2a-His_8, a chimeric dextransucrase that is fused to the carbohydrate binding module (CBM2a) of the exoglucanase/xylanase Cex (Xyn10A) of *Cellulomonas fimi* ATCC 484. Both variants are totally active and without alteration in their specificity. The DSR-F-ΔSP-ΔGBD-CBD-His_8 is purified by affinity chromatography to cellulose for the very first time.
Our results indicate that new hybrids and chimeric DSases with novel binding capacity to cellulose can be designed to obtain glyco-biocatalysts from renewable lignocellulosic materials.

Increasing circulatory half-life of a therapeutic glycoprotein with sialic acids fluorinated at C7

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Keywords: biotherapeutics, sialylation, glycoprotein, glycosylation

The use of proteins as therapeutics has revolutionized the treatment of many serious diseases. While many such therapeutics are highly effective, they can suffer from certain drawbacks including the necessity of frequent injections due to rapid clearance from the circulatory system [1]. Increasing circulatory half-life would reduce the cost and discomfort of treatment. Many therapeutic proteins are glycoproteins and the carbohydrate moieties on the proteins are known to regulate circulatory half-life. Capture by the asialoglycoprotein receptor on hepatocytes leads to internalization of proteins presenting terminal galactose residues and their removal from the circulatory system [2]. These galactose residues are exposed by the action of endogenous sialidases that cleave off terminal sialic acids. N glycans modified with hydrolysis-resistant sialic acid derivatives should confer improved circulatory half-life.

Destabilizing the oxocarbenium ion-like transition state during sialoside hydrolysis by replacing hydrogens with electron withdrawing functionalities should slow down sialic acid cleavage by sialidases. To this goal, we synthesized 7-fluorosialic acid (7-FSA)-containing glycosides and UDP-7-FSA to study the effect of this modification on rates of sialyltransferases and sialidases. In vitro kinetic measurements using model saccharides showed that sialyltransferases were largely unaffected by the modification. In contrast, hydrolysis of sialosides by several sialidases was slowed drastically.

Based on these encouraging results, the biotherapeutic α1-antitrypsin (A1AT) was engineered to carry the modified sialic acid. In a two-step procedure starting from the pharmaceutical preparation Prolastin C, A1AT was first desialylated using a bead-immobilized recombinant NedA sialidase from *Micromonospora viridifaciens* and carefully purified to ensure complete removal of the sialidase. Re-attachment of sialic acid, either the natural sugar or the 7F-analog, was performed with the sialyltransferase from *Photobacterium spec.* JT-ISH-224, which attaches sialic acids exclusively with an α2,6 linkage. The resulting engineered A1AT variants were labeled with an IR fluorophore, injected into CD-1 mice and concentration in blood determined over time [3]. Results indeed show extended half-life of the 7-FSA-modified protein compared with that to which the natural *N*-acetyl neuraminic acid had been re-attached. This therefore provides a subtle and simple way of improving the circulatory half-life of therapeutic glycoproteins.

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Combined actions of a cell surface-engineered strain of *S. cerevisiae* and LPMOs for xylitol production from Kraft pulp

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Keywords: arming yeast, consolidated bioprocessing, lignocellulose, nanofibrillation.

Industrial production of xylitol from purified D-xylose involves a costly and polluting catalytic process. Biotechnological production of xylitol from lignocellulosic waste may, therefore, constitute an advantageous option [1, 2]. In this study, xylitol was produced from Kraft pulp by using a recombinant *S. cerevisiae* YPH499 strain expressing cytosolic xylose reductase (XR), along with β-D-glucosidase (BGL), xylosidase (XYL) and xylanase (XYN) enzymes co-displayed on the cell surface, in combination with two different lytic polysaccharide monooxygenases (LPMOs) respectively from the AA9, and AA14 families [3, 4]. These two LPMOs led to a significant improvement of the xylitol production, as well as increased biomass nanofibrillation (Fig.1).

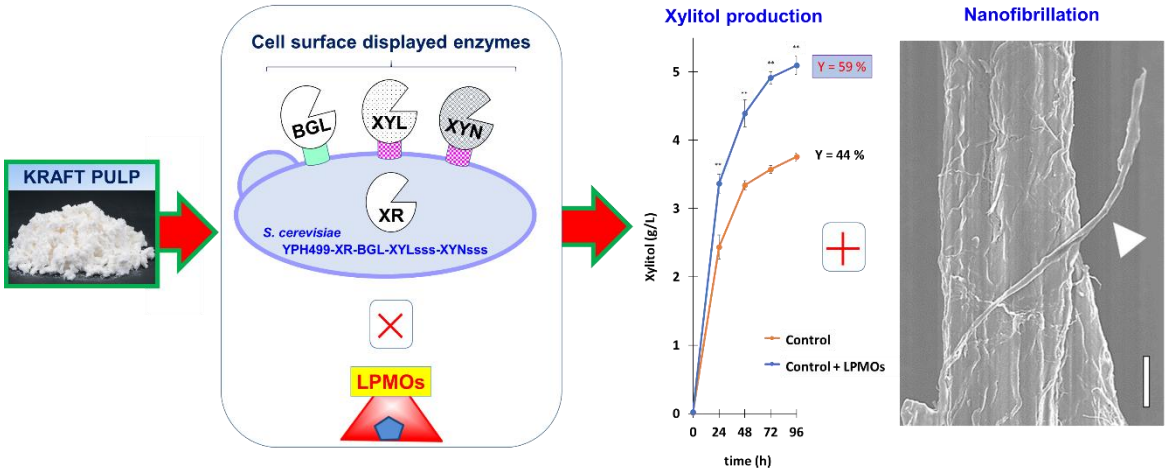

Figure 1. Schematic representation of the combined action of cell-surface engineered strain of *S. cerevisiae* and LPMOs for xylitol production and nanofibrillation of Kraft pulp. Arrow head shows a cellulose nanofibrille. Bar = 5 µm

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Developing cellulolytic *Yarrowia lipolytica* as a platform for the production of valuable products in consolidated bioprocessing of cellulose

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Keywords: *Yarrowia lipolytica*, cellulolytic biocatalyst, consolidated bioprocessing, biomass

Both industrial biotechnology and the use of cellulosic biomass as feedstock for the manufacture of various commercial goods are prominent features of the bioeconomy. With the aim to develop a consolidated bioprocess for cellulose bioconversion, we conferred cellulolytic activity to *Yarrowia lipolytica*, one of the most widely studied "nonconventional" oleaginous yeast species. Then, we exemplified how this cellulolytic *Y. lipolytica* strain can be used as a CBP platform for the production of three target products of interest: lipids, a recombinant protein, and a hydroxylated fatty acid. Considering lipid production, our results confirm that overexpression of two genes, confers the obese phenotype to the cellulolytic *Y. lipolytica*. When grown in batch conditions and minimal medium, the resulting strain consumed 12 g/L cellulose and accumulated 14% (dry cell weight) lipids. Further enhancement of lipid production was achieved either by the addition of glucose or supplementation of commercial cellulase cocktail at low enzyme loading. For the protein production, the introduction of the *LIP2* gene into cellulolytic *Y. lipolytica* led to a strain capable of producing 562 U-lipase/g-cellulose , which represents 60% of the yield obtained on glucose. Finally, expression of the hydroxylase from *Claviceps purpurea* (CpFAH12) in cellulolytic *Y. lipolytica* procured a strain that can produce ricinoleic acid (RA). Using this strain in batch cultures revealed that the consumption of 11 g/L cellulose sustained the production of 2.2 g/L RA, 69% of what was obtained on glucose.

Our work demonstrated the potential of cellulolytic *Y. lipolytica* as a microbial platform for the bioconversion of cellulose into target products. Its ability to be used in consolidated process designs has been exemplified and clues revealing how cellulose consumption can be further enhanced using commercial cellulolytic cocktails are provided.

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Marine CAZymes are key biotechnological tools for seaweed biorefinery.

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Keywords: enzyme-assisted extraction, algal polysaccharides, biorefinery.

On earth, half of the primary production occurs in the ocean, of which 25% is provided by macroalgae. About half of this biomass consists of polysaccharides, mainly sulfated, which have no equivalent in terrestrial plants.

The diversity of algal polysaccharides is considerable, consisting of storage polysaccharides such as laminarin, and cell wall polysaccharides (CWP). These CWP include neutral polysaccharides, as well as anionic (alginates) and sulfated polysaccharides (carrageenans, agars from red algae, ulvans from green algae, and fucans from brown algae).

Some of these algal polysaccharides have been already exploited as hydrocolloids. Furthermore, some of them, together with their corresponding oligosaccharides, exhibit interesting biological activities as plant biostimulants or as anti-coagulant, antiviral, anti-cancer on human cells [1,2], increasing the interest for these molecules.

Inspired by these interesting properties, we aim at producing algal extracts with applications in plant care, feed, food, cosmetics or pharmaceutics, in order to increase the value of the seaweed biomass, in a logic of biorefinery.

In this context, CAZymes with high specificities towards algal polysaccharides are useful tools for the preparation of active oligosaccharides, and also for the destructuration of algal cell wall, leading to the release of soluble molecules of interest.

The quest for such CAZymes has been at the heart of the research activity of the Marine Glycobiology Group at the Station Biologique de Roscoff for many years and resulted in a repertoire of more than a dozen enzymes, each specialized in one type of polysaccharide, with a particular specificity, and available in large amount.

In the framework of Idealg, Algolife and Genialg programs, technology transfer of enzyme production and enzyme-assisted-extraction of algal biomass is ongoing and should help implementing biorefinery networks at the regional and European levels.

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Identification and Structural Analysis of Alginate Oligosaccharide Binding Sites on β-lactoglobulin

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Keywords: β-lactoglobulin, alginate, protein-carbohydrate interaction, ITC

Polysaccharide-protein complexes are key in food texture formation and typical of certain dairy (yogurt and cheese) and other fermented foods. Previously, we characterized the interaction of two alginates (ALG) of different mannuronic acid (M)/guluronic acid (G) ratios with β-lactoglobulin (BLG) using dynamic light scattering and isothermal titration calorimetry (ITC) [1]. To gain molecular level insights on structural determinants in ALG-BLG complexes, alginate oligosaccharides (AOSs) with degree of polymerization of 3 (DP3) were generated from ALG using a *Sphingomonas sp*. lyase. The AOSs structures were determined by ^{1}H and ^{13}C NMR spectroscopy and binding sites were identified on recombinant BLG using both chemical shift perturbation of backbone resonance peaks in the ^{1}H,^{15}N-HSQC NMR spectrum at pH 2.65 and 4.0 and X-ray crystallography at pH 3.0 [2,3]. The structure of the BLG dimer in complex with AOSs at pH 3.0 and 1.75 Å resolution showed a bound D-glucuronate residue, while two other binding sites on the BLG for AOSs were identified at pH 2.65 and pH 4.0 by NMR chemical shift perturbation. These are the first structure-determined carbohydrate complexes of BLG. ITC of the AOSs gave K_d around 1 mM. The importance of the AOS composition and size was further analyzed with 9 different AOSs of DP 4–6 and defined M, G and alternating M/G content at pH 2.65 and 4.0. Interaction was also measured for technologically relevant high molecular weight ALGs (260–300 kDa) with three different M/G ratios (1.82, 1.1 and 0.55). This study can provide a basis for rational engineering of food texturizing complexes of BLG with alginate and other food hydrocolloids.

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Formation and characterization of galacto-oligosaccharides synthesized from *Lactobacillus helveticus* β-galactosidase

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Keywords: β-galactosidase, galacto-oligosaccharides, HPAEC-PAD, NMR spectroscopy

Galacto-oligosaccharides (GOS), which are non-digestible dietary carbohydrates, are an important class of prebiotics. They help to modulate the composition and activity of mammalian gut microbiome, thus conferring positive effects upon human health [1]. GOS can be enzymatically produced *via* transgalactosylation catalyzed by β-galactosidases when using lactose as a substrate. The use of GOS as a key functional ingredient is of great interest for the development of nutraceuticals and health food products [2]. Health-related benefits of GOS lead to the challenge to search for promising β-galactosidases for the production of novel GOS.

This study, hence, aimed to synthesize GOS using β-galactosidase from the food-grade bacterial strain *Lactobacillus helveticus* DSM 20075 and to structurally characterize the resulting GOS mixture. The β-galactosidase encoding genes *lacLM* from *L. helveticus* DSM 20075 were cloned and successfully expressed in *Escherichia coli* and *Lactobacillus plantarum* using pET21d and lactobacillal food-grade pSIP609 expression vectors, respectively. The purified *E.coli*βgal and the crude *L.plantarum*βgal recombinant enzymes were used for GOS synthesis. HPAEC-PAD and 1D ^{1}H NMR spectroscopy were used to determine individual GOS compounds. The continuous conversion of 600 mM initial lactose at 30 °C using recombinant β-galactosidases from *L. helveticus* rendered the maximum GOS yield of ~33% of total sugars. The increase in process temperature from 30 to 50 °C significantly decreased the reaction time from 6 to 1 h for obtaining the highest GOS yield. The enzyme showed preference to synthesize β-(1→6) and β-(1→3)-linked GOS, and the individual GOS were also determined. In conclusion, β-galactosidase from *L. helveticus* exhibits transgalactosylation property and thermostability up to 50 °C during lactose conversion, which make it attractive to industrial applications for GOS production. The information on the structure of individual GOS enhances the understanding toward the formation of these oligosaccharides using β-galactosidase from *L. helveticus*.

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Role of the KDO Glycosyltransferase KpsS in the Biosynthesis of the Polysialyltransferase Acceptor for *Escherichia coli* K1

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Keywords: KDO glycosyltransferase, capsule biosynthesis, *Neisseria meningitidis*, polysialic acid

Many pathogenic bacteria produce polysaccharide capsules to evade immune recognition and prevent lysis by the host immune response. *Neisseria meningitidis* groups B and C as well as *Escherichia coli* serogroups K1 and K92 are coated with a polysialic acid (PSA) capsule. The PSA chain is synthesized by polysialyltransferases that make an α-2,8 linkage for *E. coli* K1 and *N. meningitidis* serotype B, an α-2,9 linkage for *N. meningitidis* serotype C, or an alternating α-2,8, α-2,9 linkage for *E. coli* K92. These transferases cannot initiate synthesis of PSA *de novo*, but rather, a more complex set of machinery is required to generate an appropriate acceptor. This acceptor has been proposed to be a phosphatidylglycerol lipid anchor with a short keto-deoxyoctulosonate (KDO) linker to one or more sialic acid residues. Synthesis of this acceptor requires at least three enzymes in *E. coli* K1: KpsS, KpsC, and NeuE. In this report, we have characterized KpsS, the first enzyme in the pathway for acceptor synthesis and a membrane associated beta KDO glycosyltransferase. Much of the machinery required to produce bacterial capsules consists of transmembrane or membrane-associated enzymes. Isolation and characterization of these enzymes in an active state has proven to be challenging. Here, we have purified KpsS in a soluble and active form and have begun to investigate its function. We show that KpsS can transfer a KDO residue to a fluorescent labeled phosphatidylglycerol lipid substrate. The enzyme tolerates various lengths of lipid tails on the phosphatidylglycerol substrate, including fluorescent tags that facilitate the characterization of the substrate and product; however, the length of the tails significantly impacts the rate of the reaction. The length of the tail also impacts the binding affinity of the substrate for the enzyme. We have also shown that the enzyme can transfer modified KDO residues. Furthermore, we have isolated the product of the KpsS reaction and analyzed its structure by NMR. That KpsS is the first KDO transferase in the pathway is confirmed by the further modification of this purified product by KpsC with additional KDO units.

Tunnig phosphatidic acid and UDP-glucose metabolic pathways for glycolipids production

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Keywords: glycolipids; metabolic engineering

Glycolipids are interesting compounds due to their potential application in different fields. Especially interesting is their role as antiviral, antitumoral agents and as drug delivery systems.

The goal of this project is to produce glycoglycerolipids (GGL) in an *E. coli* platform so the complex chemical synthesis of these products could be simplified. Naturally, *E. coli* does not produce these compounds but when glycosyltransferase from *M. genitalium* MG517 is expressed in this organism, it is functional and able to produce GGL from DAG and UDP-glucose, which are common metabolites in *E .coli*. In previous studies [1] it was seen that DAG was the limiting metabolite for GGL synthesis. Therefore, different strategies to increase its pool were proposed. The first strategy was based on knocking out different genes involved in the fatty acid degradation pathway: *fadE*, a key enzyme in the ß-oxidation; *tesA gene,* a thioesterase enzyme of acyl groups and *fabR*, a DNA-binding transcriptional repressor of the fatty acid biosynthesis. Moreover, overexpression of different acyltransferases (*plsC* and *plsB*) was studied along with the overexpression of a global regulator of fatty acid biosynthesis (*fadR*), CDP-DAG pyrophosphatase (*cdh*) and phosphatidylglycerophosphatase B (*pgpB*), which was fused with PlsC to increase the DAG pool. Finally, since the availability of UDP-Glc seems to be limiting in the new constructs, overexpression of GalU and knockout of *ushA* gene, which codifies for the enzyme responsible of the reverse GalU reaction, were performed to evaluate their impact on GGL production.

[3]presented by Marc Caballé on behalf of N. Orive.

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Immobilized enzymes at work: when surface density matters

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Keywords: Immobilization, BioMolecular Welding, xylanase, enzymology.

Enzymes are involved in various type of biological processes. In many cases, they are found in multi-component machineries where enzymes are localized at close distance to each-other. The benefits of this spatial proximity on the efficiency of the enzymatic reaction are still poorly understood. We investigate this question by using an in-house developed system, Jo-In [1], where enzymes are immobilized with controlled densities - therefore distances - that can be controlled precisely. The enzyme used is a xylanase that participates to the hydrolysis of plant cell wall polymers, the Xyn11A from *Neocallimastix patriciarum* [2]. Our approach preserved the intrinsic activity of the enzyme, making the density of grafting the only parameters that is tuned.

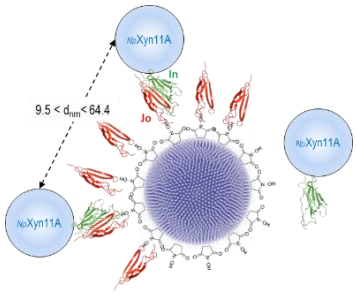

Figure 1. Tailored immobilization of xylanase Xyn11A from *Neocallimastix patriciarum via* Jo-In system to control density of grafting on solid support.

Overall, results show that xylanase molecules can be distanced from 9.5 to 64.4 nm center-to-center. Using small 4-nitrophenyl-β-D-xylotrioside as substrate, no modification of the kinetic parameters is observed compare to the enzyme in solution. However, when long polymer beechwood xylan is used as substrate, kinetic parameters are affected with higher density of grafting. The product profile was analyzed by HPAEC-PAD and MALDI-ToF. Data indicate that immobilized enzyme product profiles are different from those produced by the enzymes dispersed in solution; the immobilized enzymes release more short oligosaccharides and oligomers with average DP more homogenous. Our approach may provide some evidence that it is actually possible to control the characteristic of the products of the reaction through the immobilization of the enzymes at high surface densities. Our results questions the relationship between spatial proximity and synergistic effect as encountered in the cellulosome.

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PICT-ICEO : a platform for enzyme discovery and characterization

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Keywords: high throughput screening, enzyme engineering, biophysical characterization,

PICT-ICEO is dedicated to the screening and the characterization of original enzyme activities, isolated from directed evolution mutant protein libraries or coming from genomic or metagenomics libraries.

Technologically, PICT-ICEO focuses its resources around three competency areas: the creation of molecular diversity (genomic or metagenomics libraries, directed evolution of protein, rational or semi-rational engineering); the design and the development of original and targeted screening methods (selection tests, miniaturisation of enzymatic assays for high throughput purposes, liquid/solid medium based screening, …); the detailed study of enzyme structure/activity relationships (protein purification, crystallisation, biochemical and biophysical characterization,…). PICT-ICEO equipment therefore gathers a robotic facility for high-throughput work (liquid handling, colony picking, microplate replication), and a set of equipment for protein purification and chromatographic analysis of enzyme reaction products.

PICT-ICEO services cover:

- the high-throughput exploration of enzyme diversity, natural or artificial, involving the development of specific, original and automatized screening methods; this covers also the quantification of enzyme activities from cohorts of samples, and the isolation of genes coding for new biocatalysts.
- the comprehension of sequence/structure/function relationships of target enzymes, via rational approach or semi-rational approach, to explore protein functional landscape and go further towards the development of reliable methods to predict and engineer enzyme activity.
- the biophysical characterization of enzymes identified after selection or screening process, in order to establish a detailed biophysical mapping of proteins of interest.

A series of examples of PICT-ICEO achievements in the field of glycobiology engineering will be given to illustrate our activity.

STUDY ON THE AGGREGATION TENDENCY OF KNOWN GLYCOENZYME INHIBITORS

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Keywords: promiscuity, aggregation-based inhibitor, glycoenzyme, inhibitor specificity

Over the past few years the phenomenon of promiscuity has acquired an increasing scientific interest. Promiscuity can be defined as the significant inhibitory effect of an inhibitor on various unrelated target enzymes. It has been considered as a specifically remarkable problem in the pharmaceutical industry, since false positive hits may appear in the molecule libraries during high-throughput screening (HTS). The mechanism of such inhibitors can be based on the formation of aggregates which interact with the target protein [1-2]. In this way, we can by no means speak of specificity. The criteria of promiscuity found in literature are as follows: time-dependency, sensitivity both to enzyme concentration and detergent, increase in the particle size over time and a considerable inhibitory effect on significantly different target enzymes [3]. Despite of the fact that there are some publications in which the criteria of promiscuity are summarized, the aggregation tendency is mainly proved by applying dynamic light scattering (DLS) and not by investigating the fulfillment of the criteria. Therefore, I aimed to examine the mentioned criteria by using known glycoenzyme inhibitors to study the phenomenon of promiscuity.

The two selected inhibitors were the acarbose and a tannin, and pancreatic α-amylase was used as a model glycoenzyme in the inhibition studies. Experiments were carried out using spectrophotometry and isothermal titration calorimetry (ITC) techniques as well DLS, which allows us to monitor the possible increase in the particle size as a function of time, thus providing an unambiguous evidence for the formation of aggregates. The effect of both acarbose and tannin on the enzyme activity was assayed in the presence and absence of detergent (0.01 %Triton X-100). Moreover, inhibition measurements were accomplished in different points in time to confirm the time dependency. In the case of acarbose, with respect to the IC_{50} values, no differences could be discovered by applying detergent, various enzyme concentrations or diverse incubation time. However, there was a significant increase (approximately 55-fold growth) in the IC_{50} values of tannin (from 2.33 μg/ml to 125 μg/ml) in the presence of detergent. Based on the results of this experiment, tannin was supposed to have aggregation tendency and this assumption was confirmed by the measurements related to the time-dependency as well as by the increase in the particle size detected by DLS.

The role of the assays related to promiscuity are duel: on the one hand, by the identification of several aggregating glycoenzyme inhibitors, we may be able to discover a possible structural analogy. It could allow us to remove molecules with these properties from screening database, which could promote the greater efficiency of the HTS technique. On the other hand, if a compound has significant inhibitory effect on a number of unrelated target enzymes, but this impact is not based on the formation of aggregates, new applications of the already known drugs will be available, leading to the increase in their efficiency e.g. in complex diseases.

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A three head multi-modular protein as a versatile molecular binding platform to functionalize cellulose

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Keywords: protein engineering, multi-modularity, cellulose functionalization.

With approximatively 10^{12} tons produced yearly by photosynthesis, plant cellulose is the most abundant bio-polymers on earth [1]. Cellulose is a linear polysaccharide of D-glucose residues linked to each other *via* β-1,4 bonds. It is mainly synthetized in the plant cell wall, associated with other molecules, in order to shape plant body. However, cellulose is also secreted in the surrounding of aerobic bacteria such as *Gluconacetobacter hansenii* in order to form a bio-film enabling oxygen uptake. Nowadays, cellulose and its derivatives (nitrocellulose, cellulose acetate, methyl cellulose, carboxymethyl cellulose, etc) are among the first materials that have been intensively used in industries (wood, cotton, textiles, paper, electronics and biomedical devices) [2], representing a market size evaluated at USD 20.61 billion in 2015. By functionalizing cellulose, we aim to demonstrate how this widely used material can be more than a simple packaging or textile fibers. Based on the ability of the CBM3a from *Clostridium thermocellum* to bind to cellulose, we designed a multi-modular protein displaying three heads: the cellulose binding domain CBM3a [3], the versatile streptavidin isolated from *Streptomyces avidinii* able to bind tightly to any biotin-derivate protein [4] and the non-canonical amino acid 4-L-azidophenylalanine designed to associate specifically by click-chemistry any alkyne group containing molecule [5]. In our presentation, we will demonstrate the feasibility and the versatility of such multi-modular protein on cellulose functionalization using fluorescence and paramagnetic beads.

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Generation of a glycodiversification platform for small molecules in Escherichia coli K12

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Keywords: Glycodiversification, Polyphenols, Glycosyltransferases, Metabolic engineering

In recent years a GT toolbox (27 active GTs) has been established, with varying NDP-sugar donors, substrates and product specificities.

A modular metabolic engineering approach was performed based on *E. coli* K12(MG1655) grown on maltodextrins. The central metabolite in glycosylation is glucose 1-phosphate (G1P). A growth coupled production of G1P was developed in *E. coli* through maltodextrin utilization. *E. coli* K12 was genetically altered to increase G1P concentrations (*pgi* deletion) and natural *E. coli* NDP-sugar synthesis genes were removed (*rmlABCD*, *galU* and *glgC*) to prevent the formation of undesired glycosides.

In this research project a modular plasmid system was generated to combine the GT toolbox with the corresponding NDP-sugar synthesis genes (Glyco-switch). The GT toolbox and the NDP-switch were expressed under the control of the maltodextrin glucosidase (*malZ*) promotor, establishing an autoinduced, modular expression system.

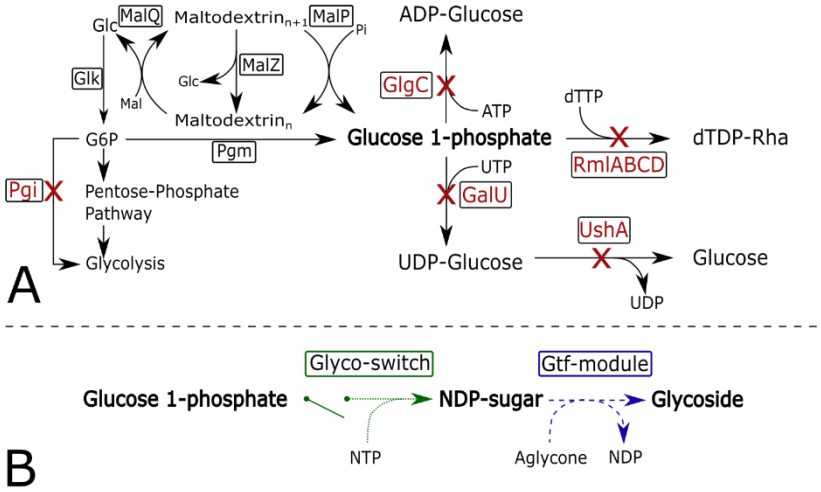

Figure 1. **A.** Metabolic engineering of E. coli **B.** Modular approach to glycosylation

The potential of the newly developed glycosylation plattform was demonstrated using naringenin as substrate. GTW (derived from *Bacillus weihenstephanensis* HH3) combined with the *galU* expressing NDP-switch was able to produce 4.8 g/L Naringenin 7-O-glucoside, whereas GTD (derived from *Dyadobacter fermentans*) with *rmlABCD* expressing NDP-switch was able to produce 13.1 g/L Naringenin 5-O-rhamnoside in 72 hrs biotransforamtions.

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Immobilization of β-galactosidase on electrospun gelatin mats for the production of novel prebiotic oligosaccharides

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Keywords: electrospinning, β-gal immobilization, galactooligosaccharides, sweeteners

The worldwide sugar intake strongly increased in the last decades partly due to consumption of high caloric, sweet beverages resulting in obesity, cardiovascular diseases, and dental caries [1]. To improve the host's health and well-being, substituting sugar with dietary, prebiotic oligosaccharides, that selectively stimulate the growth of the gastrointestinal microflora, can be beneficial, especially if those components are of sweet taste. Galactooligosaccharides (GOS) can be synthesized via transgalactosylation by the enzyme β-galactosi-dase (β-gal) (EC 3.2.1.23), where the galactosyl moiety of lactose is transferred to a nucleophilic acceptor to produce GOS with a huge diversity in structure. New acceptors may create an alternative to sugar in dairy products by simultaneously eliminating lactose making these functional dairy foods interesting for lactose intolerant people. To make the production of novel sweet-oligosaccharides economically more attractive, it is advantageous to immobilize β-gal on a suitable, hazard-free carrier material.

Electrospinning features a simple and easily reproducible method for fabricating fine nanofiber mats from polymer solutions with enormous surface-to-mass ratios, extensive convertibility, and easy handling thus making them excellent for the enzyme immobilization.

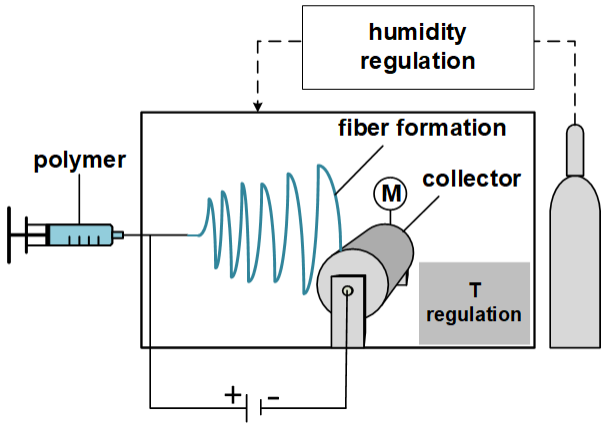

Figure 1: Schematic horizontal electrospinning setup in a climate control chamber with external regulation of humidity and temperature.

The aim of this study was to immobilize β-gal from *Aspergillus oryzae* on electrospun gelatin fiber mats via two methods (covalent binding and entrapment). Immobilization parameters were optimized and operational stability as well as industrial feasibility were investigated. Sucralose, neohesperidine dihydrochalcone and aspartame were tested as promising β-gal acceptors and the novel, sweet GOS were characterized.

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