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Investigating synergism within multimodular glycoside hydrolases during wheat straw cell wall deconstruction

Cédric Montanier, Louise Badruna, Thierry Vernet, Anne-Marie Di Guilmi, Vincent Burlat, Michael O'Donohue

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11th

Carbohydrate

Bioengineering Meeting



10-13 May 2015, Espoo, Finland



ABSTRACTS

Welcome to CBM11

The eleventh Carbohydrate Bioengineering Meeting (CBM11) focuses on various aspects of carbohydrate-acting enzymes and biomolecules, their mode of action, structure and structure function relationships, engineering and applications. The applications spread from health and nutrition to material sciences. The subjects are highly topical in the present world, where sustainable use of renewables is a key interest, not just for scientists, but for the whole society, governments and industries. Thanks to the contributions of the participants we have been able to design a programme which covers the recent developments in the focus areas of the meeting. The present programme in Espoo includes 55 talks and flash presentations and 133 poster presentations.

The roots of CBM11 go back to Helsingør, Denmark and 1995, when the first CBM meeting was organized twenty years ago. The major aim of the meeting is again to bring together colleagues and scientists active in various fields of carbohydrates, biomolecules and carbohydrate-active enzymes. Our target has been to generate a pleasant environment for knowledge sharing, generating collaborations, and hopefully also for constituting an incubator for many future project ideas.

The meeting takes place in Otaniemi, in a beautiful natural cape of the Baltic Sea in Espoo, the home of Aalto University engineering schools and VTT. The programme runs in Dipoli, a famous conference building designed in early 1960's by Raili and Reima Pietilä showing the interplay of light, Finnish pine wood, copper, and natural rocks. If you are interested in architecture, you may want to take a walk in the campus area, which is designed by the most famous Finnish architect Alvar Aalto and to stop by in the main building of Aalto University and the main library, both from 1960's.

On the behalf of the local Organizing Committee we would like to express our gratitude to all our sponsor companies and to all persons who have contributed to organizing this meeting.

We wish you a fruitful meeting of scientific excellence and enjoyable stay in spring-like Espoo and Finland.

Kristiina Hilden
Anu Koivula
Kristiina Kruus
Markus Linder
Maija Tenkanen

Carbohydrate Bioengineering Meeting History

CBM1	1995	Elsinore, Denmark
CBM2	1997	La Rochelle, France
CBM3	1999	Newcastle, United Kingdom
CBM4	2001	Stockholm, Sweden
CBM5	2003	Groningen, The Netherlands
CBM6	2005	Barcelona, Spain
CBM7	2007	Braunschweig, Germany
CBM8	2009	Ischia, Naples, Italy
CBM9	2011	Lisbon, Portugal
CBM10	2013	Prague, Czech Republic

International Programme Committee

Birte Svensson (chair)	Technical University of Denmark, Denmark
Vincent Bulone	Royal Institute of Technology, Stockholm, Sweden
Pedro Coutinho	CNRS, Aix-Marseille Université, France
Gideon J. Davies	University of York, United Kingdom
Lubbert Dijkhuizen	University of Groningham, the Netherlands
Ten Feizi	Imperial College, London, United Kingdom
Carlos Fontes	Technical University of Lisbon, Portugal
Vladimir Kren	Academy of Sciences of the Czech Republic, Czech Republic
Takashi Kuriki	Ezaki Glico Co. Ltd., Osaka, Japan
Marco Moracci	CNR, Naples, Italy
Carsten Andersen	Novozymes A/S, Copenhagen, Denmark
Antoni Planas	Universitat Ramon Lull, Barcelona, Spain
Magali Remaud-Simeon	INSA, Toulouse, France
Steve G. Withers	University of British Columbia, Vancouver, Canada

Local Organizing Committee

Maija Tenkanen (chair)	University of Helsinki, Finland
Kristiina Hilden	University of Helsinki, Finland
Anu Koivula	VTT, Finland
Kristiina Kruus	VTT, Finland
Markus Linder	Aalto University, Finland

11th Carbohydrate Bioengineering Meeting

10-13 May, 2015

Espoo, Finland



Programme

Sunday 10.5.2015

16.00 **Opening of CBM11**

Chair: Birte Svensson

16.05 **Welcome speech**

President Tuula Teeri
Aalto University, Finland

16.20 **Opening lecture**

T1: From the first CBHI to biorefineries

Merja Penttilä
VTT, Finland

Glycomics, systems glycobiology and bioinformatics

Chair: Markus Linder

17.00 **T2: CAZyChip: a bioChip for bacterial glycoside hydrolases detection and dynamic exploration of microbial diversity for plant cell wall hydrolysis**

Claire Dumon
Université de Toulouse, France

17.20 **T3: A new generation of chromogenic substrates for high-throughput screening of glycosyl hydrolases, LPMOs and proteases**

Julia Schückel
University of Copenhagen, Denmark

17.40 **T4: Mining fungal diversity for novel carbohydrate acting enzymes**

Ronald P. de Vries
Utrecht University, The Netherlands

18.00 **End of the day**

19 – 21 **Get-together, Design Factory**

Monday 11.5.2015

Mechanisms of carbohydrate-acting enzymes I

Chair: Takashi Kuriki

- 9.00 Key-note lecture**
T5: The increasing diversity of lytic polysaccharide monoxygenases
Gideon Davies
University of York, UK
- 9.40 T6: Neutron and high-resolution X-ray structural studies of glycoside hydrolase family 45 endoglucanase from the basidiomycete *Phanerochaete chrysosporium***
Kiyohiko Igarashi
University of Tokyo, Japan
- 10.00 T7: New insight into substrate specificity and activity determinants of a starch debranching enzyme gained from substrate: enzyme crystal structures**
Marie S. Møller
Carlsberg Laboratory, Denmark
- 10.20 T8: Crystal structures of N-acetylhexosamine 1-kinase and UDP-glucose 4-epimerase in the GNB/LNB pathway from infant-gut associated bifidobacteria**
Shinya Fushinobu
University of Tokyo, Japan
- 10.40 Coffee break and poster viewing**

Mechanisms of carbohydrate-acting enzymes II

Chair: Anu Koivula

- 11.20 T9: Crystal structure of the GTFB enzyme, the first representative of the 4,6- α -glucanotransferase subfamily within GH70**
Tjaard Pijning
University of Groningen, The Netherlands
- 11.40 T10: Catalytic mechanism of retaining glycosyltransferases: Is Arg293 on the β -face of EXTL2 compatible with it? Insights from QM/MM calculations**
Laura Masgrau
Universitat Autònoma de Barcelona, Spain
- 12.00 T11: Structure-function studies of enzymes in the oxidative D-galacturonate pathway**
Helena Taberman
University of Eastern Finland, Finland
- 12.20 Flash presentations (5 min each)**
Chair: Antoni Planas
- P15: A single point mutation near the active center is responsible for high efficiency of the *Thermotoga maritima* α -galactosynthase in the synthesis of known amylase substrate**
Kirill Bobrov
B.P.Konstantinov Petersburg Nuclear Physics Institute, Russia

P16: Insights into LPMO diversity from structural and functional characterization of NcLPMO9C, a broad-specificity lytic polysaccharide monoxygenase

Anna S. Borisova

Swedish University of Agricultural Sciences, Sweden.

P32: Assisting effect of a carbohydrate binding module on glycosynthase-catalyzed polymerization

Magda Faijes

Universitat Ramon Llull, Spain

P43: Rational design of a novel cyclodextrin glucanotransferase from *Carboxydocella* to improve alkyl glycoside synthesis

Kazi Zubaida Gulshan Ara

Lund University, Sweden

P44: Development and application of a synthetic cellulosome-based screening platform for enhanced enzyme discovery

Johnnie Hahm

Novozymes, Inc. USA

P49: Neopullulanase subfamily and related specificities of the family GH13 - *in silico* study focused on domain evolution

Stefan Janecek

Slovak Academy of Sciences, Slovakia

P50: Characterization of a GH30 glucuronoxylan specific xylanase from *Streptomyces turgidiscabies* C56

Satoshi Kaneko

University of the Ryukyus, Japan

P53: Solution structures of glycosaminoglycans and their complexes with complement Factor H: implications for disease

Sanaullah Khan

University College London, UK

13.00 Lunch

Carbohydrates in health

Chair: Vladimir Kren

14.20 Key-note lecture

T12: Polysaccharide engineering: towards carbohydrate drugs and drug carriers

Takeshi Takaha

Ezaki Glico Co., Ltd. Japan

15.00 T13: Structure and mechanism of action of O-acetyltransferase (Oat) A

Anthony J. Clarke

University of Guelph, Canada

15.20 T14: Complete switch from α 2,3- to α 2,6-regioselectivity in *Pasteurella dagmatis* β -D-galactoside sialyltransferase by active-site redesign

Katharina Schmölzer

Austrian Centre of Industrial Biotechnology, Austria

15.40 **Poster session and coffee**

Carbohydrate and enzyme engineering I

Chair: Pedro Coutinho

16.40 **T15: Structure and function in the GH53 β -1,4-galactanase family**

Leila Lo Leggio

University of Copenhagen, Denmark

17.00 **T16: Determinants of substrate specificity in chitin oligosaccharide deacetylases: How loops define the de-N-acetylation pattern**

Antoni Planas

Universitat Ramon Llull, Spain

17.20 **T17: Molecular basis for the epimerization of oligosaccharides by cellobiose 2-epimerase**

Wataru Saburi

Hokkaido University, Japan

17.40 **End of the day**

Tuesday 12.5.2015

Structure-function relationships of carbohydrate-acting enzymes I

Chair: Carsten Andersen

9.00 **Key-note lecture**

T18: Sugar oxidoreductions at the crossroads of mechanistic enzymology and biotechnological application

Bernd Nidetzky

Technische Universität Graz, Austria

9.40 **T19: Functional characterization of a set of fungal lytic polysaccharide monooxygenase secreted by *Podospora anserina***

Chloé Bennati-Granier

INRA, France

10.00 **T20: Glucoligosaccharide oxidases: Determinants of activity and use in carbohydrate modification**

Emma R. Master

University of Toronto, Canada

10.20 **T21: Engineering of pyranose oxidoreductases for bio-fuelcell applications**

Clemens Peterbauer,

University of Natural Resources and Life Sciences Vienna, Austria

10.40 **Coffee break and poster viewing**

Structure-function relationships of carbohydrate-acting enzymes II

Chair: Kristiina Hilden

- 11.20 **T22: The role of carbon starvation in the induction of enzymes that degrade plant-derived carbohydrates in *Aspergillus niger***
Jolanda van Munster
University of Nottingham, UK
- 11.40 **T23: Esterases of *Myceliophthora thermophila* C1 help in the degradation and modification of lignocellulosic material**
Laura Leonov
Dyadic Nederland BV, The Netherlands
- 12.00 **T24: Processive action of *Rasamsonia emersonii* cellobiohydrolase Cel7A**
Anu Koivula
VTT, Finland
- 12.20 **T25: Hydrolysis of arabinoxylo-oligosaccharides and wheat flour arabinoxylan by α -L-arabinofuranosidases**
Vincent McKie
Megazymes, Ireland
- 12.30 **Flash presentations (5 min each)**
Chair: Kristiina Kruus
- P60: Variations in the substrate specificity of cellobiose dehydrogenase**
Daniel Kracher
University of Natural Resources and Life Sciences, Vienna, Austria
- P71: Structural and functional insights on the glycoside hydrolases involved in the metabolism of xylooligo- and arabinooligosaccharides in lactic acid bacteria**
Javier A. Linares-Pastén
Lund University, Sweden
- P93: Diversity of xylan deacetylases of family CE16: action on acetylated aldotetrauronic acid and glucuronoxylan**
Vladimir Puchart
Slovak Academy of Sciences, Slovakia
- P94: Conformational studies on trivalent acetylated mannanose clusters**
Jani Rahkila
Åbo Akademi University, Finland
- P130: Expression a hyperthermostable *Thermotoga maritima* xylanase 10B in *Pichia pastoris* GS115 and its tolerance to ionic liquids**
Hairong Xiong
College of Life Science, China
- P132: Reconstruction of genome-scale metabolic model of *Brevibacillus thermoruber* 423 for design of improved EPS production strategies**
Songul Yasar Yildiz
Marmara University, Turkey
- 13.00 **Lunch**

Synthesis, structure and function of carbohydrates and glycoconjugates

Chair: Vincent Bulone

- 14.20 Key-note lecture**
T26: Exploring plant cell wall xylan biosynthesis, structure and function
Paul Dupree
University of Cambridge, UK
- 15.00 T27: Understanding the effect of overexpression of fungal acetyl xylan esterase (AXE1) in hybrid aspen**
Prashant Mohan-Anupama Pawar
Swedish University of Agricultural Sciences, Sweden
- 15.20 T28: Bioinspired model assemblies of plant cell walls as sensors for unravelling interaction features of CAZymes**
Gabriel Paës
INRA and University of Reims Champagne-Ardenne, France
- 15.40 Poster session and coffee**
- Materials from renewable carbohydrates***
Chair: Maija Tenkanen
- 16.40 T29: Discovery of original α -transglucosylases from *Leuconostoc citreum* NRRL B-1299 and NRRL B-742 for the synthesis of tailor-made α -glucans**
Claire Moulis
Université de Toulouse, France
- 17.00 T30: Marine-derived bacterial polysaccharides are valuable sources of glycosaminoglycans**
Lou Lebellenger
Centre Atlantique, rue de l'Île d'Yeu, France
- 17.20 T31: Spider silk mimicking assembly of nanocellulose**
Sanni Voutilainen
Aalto University, Finland
- 17.40 End of the day**
- 19.30 Conference dinner, Restaurant Pörssi (downtown Helsinki)**

Wednesday 13.5.2015

Carbohydrate and enzyme engineering II

Chair: Magali Remaud-Simeon

- 9.00 Key-note lecture**
T32: Multiple CBMs enhance starch degradation by members of the human gut microbiota
Nicole Koropatkin
University of Michigan, USA
- 9.40 T33: Functionality of granule-bound starch synthase from the waxy barley cultivar CDC Alamo**
Kim H. Hebelstrup
Aarhus University, Denmark
- 10.00 T34: Glucan phosphatases utilize different mechanisms to bind starch and glycogen**
Matthew S. Gentry
University of Kentucky, USA
- 10.20 T35: Secondary structure reshuffling modulates the enzymatic activity of a GT-B glycosyltransferase at the membrane interface**
Natalia Comino
Universidad del País Vasco / Euskal Herriko Unibertsitatea, Spain
- 10.40 T36: Degrading sulfated sugars from the sea: novel insights into the evolution, dimerization plasticity and catalytic mechanism of the GH117s**
Elizabeth Ficko-Blean
Sorbonne Universités, France
- 11.00 Coffee break**

Carbohydrates in nutrition

Chair: Marco Moracci

- 11.40 T37: Functional metagenomics reveals novel pathways of mannoside metabolism by human gut bacteria**
Gabrielle Potocki-Veronese
Université de Toulouse, France
- 12.00 T38: Structural basis for arabinoxylo-oligosaccharide capture by probiotic bifidobacteria**
Maher Abou Hachem
Technical University of Denmark, Denmark
- 12.20 T39: The modular intramolecular trans-sialidase from *Ruminococcus gnavus* ATCC 29149 suggests a novel mechanism of mucosal adaptation in the human gut microbiota**
Louise E Tailford
Institute of Food Research, UK
- 12.40 T40: Galactomannan degradation by *Bifidobacterium***
Evelina Kulcinskaja
Lund University, Sweden

Chair: Lubbert Dijkhuizen

- 13.00** **Closing lecture**
T41: Glycan utilization by human gut Bacteroides
Harry Gilbert
University of Newcastle upon Tyne, UK
- 13.40** **Poster awards, closing and invitation to CBM12**
- 14.00** ***End of CBM11***
- Lunch**

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Oral Presentations

From the first CBHI to biorefineries

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CAZyChip: a bioChip for bacterial glycoside hydrolases detection and dynamic exploration of microbial diversity for plant cell wall hydrolysis

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The development of biocatalysts for the deconstruction of plant cell wall polysaccharides such as cellulose and hemicellulose is currently a major endeavor and will contribute to the development of the bioeconomy.

Micro-organisms play an important role in biotransformation of plant cell walls because they produce large collections of enzymes, including glycoside hydrolases (GHs) that are key enzymes involved in the deconstruction of plant cell wall polysaccharides. In order to explore and elucidate the functional dynamic of microbial communities degrading plant cell wall, we developed a robust and generic tool, the CAZyChip based on DNA microarray containing all the bacterial GH classified in the CAZy database. This chip allows a rapid characterization of GH at transcriptomic level and the characterization of plant cell wall-degrading enzyme systems that act in concert on the different polysaccharide components of lignocellulosic biomass. The custom microarray was tested and validated by the hybridization of GHs RNA extracted from *E. coli* and recombinant *E. coli* strains. Our results suggest that a microarray-based study can detect genes from low-expression in bacteria. In addition, the results of hybridization of complex biological samples such as rumen or termite gut will be presented. The CAZyChip appears to be an effective tool for profiling GH expression in microbial communities that are actively degrading lignocellulosic biomass and could guide the design of enzymatic cocktails.

A new generation of chromogenic substrates for high-throughput screening of glycosyl hydrolases, LPMOs and proteases

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Enzymes that degrade or modify polysaccharides are widespread in pro- and eukaryotes and have multiple biological roles and biotechnological applications. Recent advances in genome and secretome sequencing, together with associated bioinformatic tools have enabled large numbers of putative carbohydrate acting enzymes to be putatively identified. However, there is a paucity of methods for rapidly screening the activities of these enzymes and this is serious bottleneck in the development of enzyme-reliant bio-refining processes. We have developed a new generation of multi-coloured chromogenic polysaccharide and protein substrates that can be used in cheap, convenient and high-throughput multiplexed assays. In addition we have produced substrates of biomass materials in which the complexity of plant cell walls is partially maintained. We show that these substrates can be used to screen the activities of glycosyl hydrolases, lytic polysaccharide monoxygenases (LPMOs) and proteases, and provide insight into substrate availability within biomass. We have validated the technique using microbial enzymes and further show here that these new assays enable the rapid analysis of endogenous enzymes in diverse plant materials.

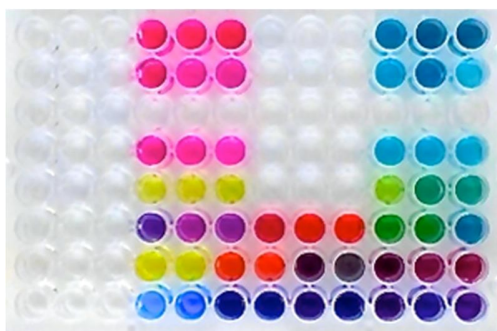


Fig 1: Product plate of a multiplexed assay of different chromogenic polysaccharide hydrogel (CPH) substrates treated with different enzymes.

Mining fungal diversity for novel carbohydrate acting enzymes

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The availability of fungal genome sequences has provided a wealth of new genes and their corresponding enzymes as candidates for novel or better biocatalysts. In particular with respect to enzymes acting on plant biomass, the differences in genome content in the fungal kingdom is enormous and can to a certain extent be related to the natural biotope of the species.

So how do we find the most promising enzymes or enzyme sets from this near limitless pool of candidates? Modern bioinformatics tools can provide the answer to this question, but only in combination with extensive biological data sets that provide insight into the relevance of genomic differences. Extensive transcriptome and proteome datasets on fungi growing on diverse carbon sources, including crude plant biomass as well as pure components thereof allow identification of the enzymes that are required for these different substrates. Comparative genomics and transcriptomics can identify crucial enzymes by selecting those that are present in a large variety of fungi, while phylogeny can pinpoint enzymes that more likely have different properties or substrate specificities.

In this presentation I will provide examples how promising candidate enzymes and enzyme sets can be discovered by combining comparative genomics, transcriptomics and proteomics with growth profiling and data about fungal biotopes and enzymatic function.

The increasing diversity of lytic polysaccharide monooxygenases

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Lytic Polysaccharide Monooxygenases are establishing themselves as important players on biomass conversion (recently reviewed in Refs^{1,2}). These mononuclear copper containing enzymes now form four distinct families in the CAZY classification (AA9 and AA10, formally known as GH61 and CBM33) as well as the recently discovered AA11³ and AA13^{4,5} families. Whilst most LPMO families are active on beta-linked polysaccharides, the first starch-active LPMO family has also recently been described and characterised.^{4,5} In this lecture I will summarize the LPMO field, highlighting recent work by the CESBIC consortium (University of York, University of Cambridge, University of Copenhagen, CNRS Marseille, and Novozymes A/S) notably in the area of enzyme discovery and characterisation^{3,4} of the reactive Cu centre.⁶

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Neutron and high-resolution X-ray structural studies of glycoside hydrolase family 45 endoglucanase from the basidiomycete *Phanerochaete chrysosporium*

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We employed a neutron diffraction analysis to investigate the catalytic mechanism of the inverting glycosidase hydrolase (GH) family 45 cellulase *PcCel45A*, which is an endoglucanase (EG) belonging to subfamily C of this family, isolated from the basidiomycete *Phanerochaete chrysosporium*. The amino acid alignment with other GH family 45 EGs indicates *PcCel45A* lacks putative general base and assisting acidic residues while it has an apparent activity towards cellulose and β -1,3-1,4-glucan (1). To understand the catalytic mechanism of *PcCel45A*, we made a large crystal of 6 mm³ volume (3 mm x 2 mm x 1 mm) for the neutron protein structural study (2). The results of a joint refinement of the neutron and high-resolution X-ray structures clarified a key role of tautomerization of asparagine 92 to imidic acid as a catalytic base in the inverting cellulase.

Acknowledgments

We thank Profs. Katsuhiro Kusaka, Taro Yamada, Ichiro Tanaka, Nobuo Niimura in Ibaraki University, Prof. Shinya Fushinobu in the University of Tokyo, Prof. Satoshi Kaneko in the University of Ryukyus, Dr. Kazunori Ohta in Space Environment Utilization Center, Japan Aerospace Exploration Agency, Dr. Hiroaki Tanaka in Confocal Science Inc., Dr. Koji Inaka in Maruwa Foods and Biosciences Inc., and Prof. Yoshiaki Higuchi in University of Hyogo for their contributions to this study.

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New insight into substrate specificity and activity determinants of a starch debranching enzyme gained from substrate:enzyme crystal structures

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Pullulanases are industrially important starch debranching enzymes and the mechanisms driving their substrate specificities and activities can have a direct influence on the profit ratio in e.g. the industrial manufacturing of glucose and syrups from starch. To date crystal structures of type I pullulanases from 7 different organisms have been solved, including the barley limit dextrinase (LD). Some of these enzyme structures are solved in complex with hydrolysis products or inhibitors, but none of the pullulanases have been structure determined in complex with a natural substrate, i.e. an α -1,6-branched maltooligosaccharide. Here we present crystal structures of inactive LD in complex with 1) a limit dextrin (PDB 4J3W), and 2) with a pullulan derivative (PDB 4J3X) [1]. These are the first type I pullulanase structures with intact α -1,6-glucosidic linked substrates spanning the active site. Together with the structures of LD and bacterial pullulanases in complex with hydrolysis products they are used for suggesting both a mechanism for nucleophilicity enhancement in the active site as well as a mechanism for avoidance of dual α -1,6- and α -1,4-hydrolytic activity likely to be a biological necessity during starch synthesis, where LD has a role in trimming of branches.

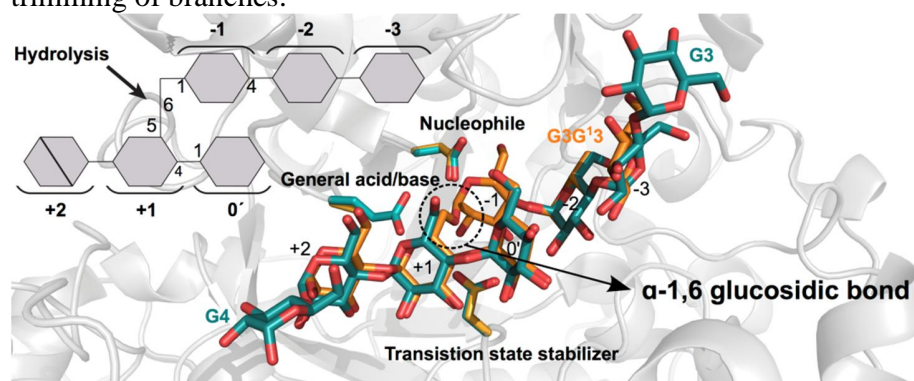


Fig 1. Superimposition of barley limit dextrinase in complex with a limit dextrin/branched maltooligosaccharide substrate (G3G'3; colored in orange) and linear products (maltotriose and maltotetraose; coloured in teal), respectively.

Acknowledgements: Access to synchrotron beam lines was made possible through the support from DANSCATT. We thank MAX II Laboratory, ESRF and the associated staff for beam time and assistance.

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Crystal structures of N-acetylhexosamine 1-kinase and UDP-glucose 4-epimerase in the GNB/LNB pathway from infant-gut associated bifidobacteria

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Infant-gut associated Bifidobacteria have a metabolic pathway specific for disaccharides liberated from human milk oligosaccharides (Gal- β 1,3-GlcNAc, lacto-*N*-biose I, LNB) and intestinal mucin glycans (Gal- β 1,3-GalNAc, galacto-*N*-biose, GNB) (Fig. 1A) [1]. The pathway consists of four intracellular enzymes including *N*-acetylhexosamine 1-kinase (NahK) and UDP-glucose 4-epimerase (GalE) [2]. NahK is an anomeric kinase that can produce various sugar 1-phosphates [3]. GalE has wide substrate specificity and epimerizes both UDP-Glc/Gal and UDP-GlcNAc/GalNAc. We have determined the crystal structures of NahK (Fig. 1B) and GalE (Fig. 1C) from *Bifidobacterium longum* JCM1217. Structural bases for the substrate recognition, catalysis, and ligand-induced movement of these enzymes were revealed.

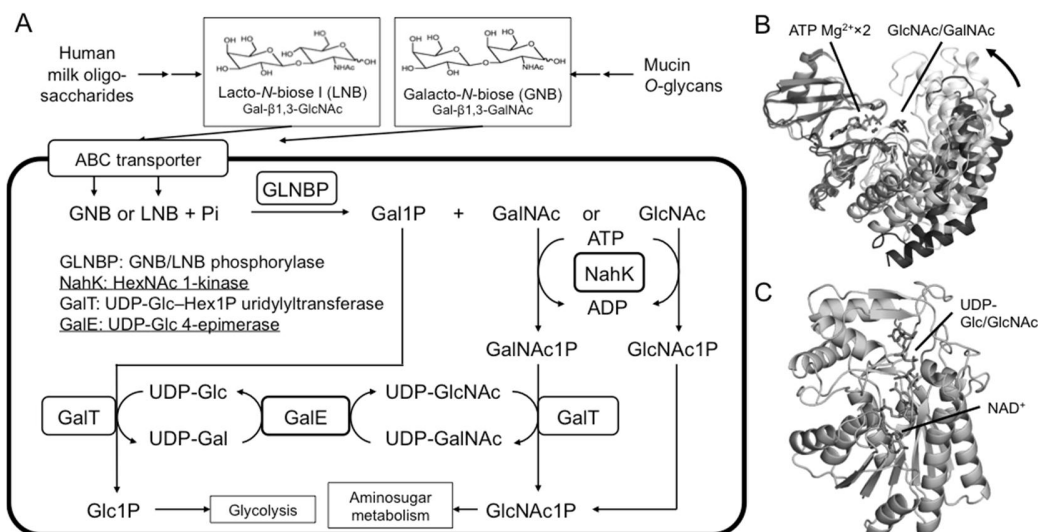


Fig 1. The GNB/LNB pathway (A) and crystal structures of NahK (B) and GalE (C).

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Crystal structure of the GTFB enzyme, the first representative of the 4,6- α -glucanotransferase subfamily within GH70

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Within the glycoside hydrolase family GH70, glucansucrases utilize sucrose to synthesize a variety of α -glucan polymers¹. Recently a subfamily within GH70 was described^{2,3}, containing enzymes highly homologous to glucansucrases but inactive on sucrose. Instead, these enzymes utilize malto-oligosaccharides and starch as glucose donor substrates for α -glucan synthesis, acting as 4,6- α -glucanotransferases. The linear oligosaccharide products are rich in α -1,6 glycosidic linkages⁴, and provide an exciting type of carbohydrate for the food industry, acting as prebiotics and providing a soluble fiber.

In this work we determined the 3D atomic structure of GTFB, a GH70 4,6- α -glucanotransferase from *Lactobacillus reuteri* 121, using a construct (GTFB- Δ N Δ V) comprising the catalytic domain A as well as domains B, C and IV. The crystal structure of GTFB- Δ N Δ V at 1.80 Å (Fig. 1.) allowed us to compare the different specificities within GH70 and to obtain insights in the unique reaction mechanism of 4,6- α -glucanotransferases, which may represent an evolutionary intermediate between the GH13 and GH70 enzyme families.

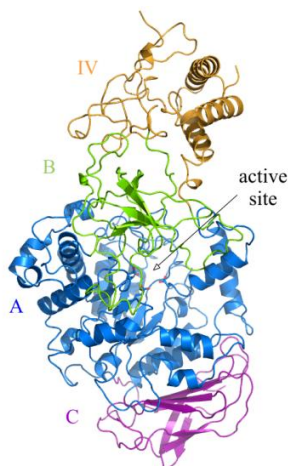


Fig 1. Crystal structure of GTFB- Δ N Δ V with the domains and location of the active site indicated.

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Catalytic mechanism of retaining glycosyltransferases: Is Arg293 on the β -face of EXTL2 compatible with it? Insights from QM/MM calculations

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The synthesis of pure glycans, and in sufficient quantities, is highly pursued to help the development of glycosciences and related applications. In Nature, glycosyltransferases (GTs) are responsible for their biosynthesis. The catalytic mechanism of GTs, and of retaining GTs in particular, has been under debate for long. In the last years, computational studies have brought light into the discussion. Nevertheless, all the proposed mechanisms involve the formation of oxocarbenium species, either as a short-lived ion-pair intermediate or as a transition state, which hold the development of a positive charge density at the anomeric centre (Fig 1).

In that sense, the active site of retaining α 1,4-N-acetylhexosaminyltransferase (EXTL2), with a positively charged residue (R293) at close proximity of the anomeric carbon, is puzzling. Does EXTL2 open the door for a new class of mechanism in retaining GTs?

Our goal here has been to evaluate whether the presence of R293 in EXTL2 is compatible with the front-side attack mechanism or whether a different mechanism must be proposed, and to reveal the role of this residue in such position.¹ The results are discussed in the light of what we have learned in the last years about the catalytic mechanism of retaining glycosyltransferases.²⁻⁵

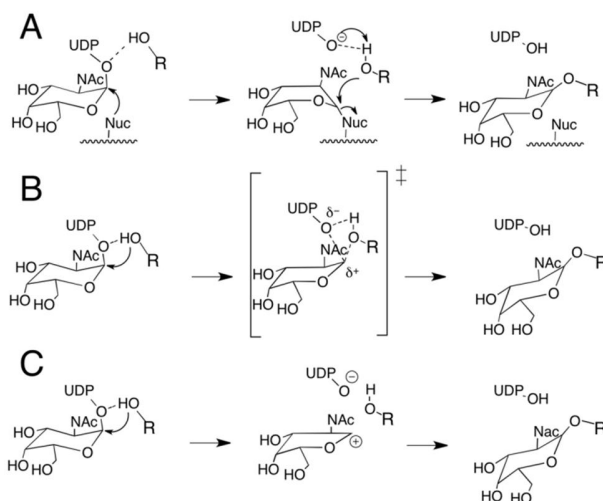


Fig 1. Proposed mechanisms for retaining glycosyltransferases. (A) Double-displacement with formation of a glycosyl-enzyme intermediate, and front-side attack via oxocarbenium (B) transition state or (C) ion pair intermediate.

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Structure-function studies of enzymes in the oxidative D-galacturonate pathway

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Plant cell wall polysaccharides cellulose, hemicellulose and pectin constitute the major fraction of the lignocellulosic feedstock, and provide the raw material for microbial conversion to fuels and chemicals. Glucose has been the most studied and applied monosaccharide source, but in order to make the biorefining concepts more economically feasible, it is desirable to utilize also the less explored biomass-derived sugars.

Pectin is mainly composed of D-galacturonate, a sugar acid that is used as a carbon and energy source by many bacterial and fungal sources. D-galacturonate has two known catabolic routes in bacteria: the isomerase and the oxidative pathway. The oxidative pathway has been shown to be active in *Agrobacterium tumefaciens* and *Pseudomonas syringae*. In the oxidative pathway in *A. tumefaciens* D-galacturonate is first oxidized by uronate dehydrogenase (*At Udh*) [1] to D-galactaro-1,5-lactone, which is then isomerised to D-galactaro-1,4-lactone either non-enzymatically or by D-galactarolactone isomerase [2]. A novel galactarolactone cycloisomerase (*At Gci*) then catalyses the ring opening into 3-deoxy-2-keto-hexarate [3], which is converted further to α -ketoglutaric semialdehyde by keto-deoxy-D-galactarate dehydratase (*At KDG*) [4, 5]. Finally, α -ketoglutaric semialdehyde is oxidized by a dehydrogenase to α -ketoglutarate, which is a metabolite of the TCA cycle [6].

The structures of *At Udh*, *At Gci* and *At KDG* dehydratase and their complexes have been solved by X-ray crystallography [1, 5, 7]. Structure-function studies are crucial for a comprehensive understanding of the microbial oxidative D-galacturonate pathway and its applications in sustainable chemical production.

Acknowledgements The work has been supported by the National Doctoral Programme in Informational and Structural Biology, and the Finnish Centre of Excellence in White Biotechnology-Green Chemistry programme (Academy of Finland decision number 118573).

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Polysaccharide engineering: towards carbohydrate drugs and drug carriers

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Bio-macromolecules (e.g. protein, peptide, antibody, DNA or RNA) present in our body is now widely utilized in pharmaceuticals. Carbohydrates, on the other hand, received increasing attention as drug candidates, but carbohydrates used for pharmaceuticals is limited to few glycosaminoglycans (heparin, hyaluronan, chondroitin sulfate).

Ezaki Glico have been working in the field of carbohydrate bioengineering, and aimed to develop new business (products, materials), especially for health and nutrition, from basic findings. These products include several key-enzymes for carbohydrate bioengineering (branching enzyme, amyloamylase, glucoamylase, glucoamylase, sucrose phosphorylase and amylase), phosphoryl oligosaccharides of calcium¹), cyclic glucans (cycloamylose²) and cluster dextrin³), synthetic polysaccharides (amylose and glycogen⁴) and glycosides⁵). These materials have been used in food, cosmetic, pharmaceutical, and other industries. We are currently challenging to combine all our resources to develop a versatile platform for carbohydrate drugs and drug carriers.

Glycogen is a predominant polysaccharide in our body with very attractive structure and characteristics. It is a single molecular nano-sized spherical particle with dendritic architecture where numerous non-reducing end glucoses constitute the surface. We have developed enzymatic system to produce artificial glycogen (GD) with strictly controlled particle size. GD are further subjected to non-reducing end specific glycosylation technology where GD surface is modified with various sugar moieties. Surface engineered GD is a novel and versatile platform for carbohydrate drugs and drug carriers.

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Structure and mechanism of action of O-acetyltransferase (Oat) A

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Variations in the chemical structure of peptidoglycan (PG) contribute to resistance to the action of both the innate immune system of host organisms and antibiotics. For example, PG O-acetyltransferase (Oat) A is responsible for the O-acetylation of the C-6 hydroxyl group of N-acetylmuramoyl residues in the PG of Gram-positive bacteria and deletion of the *oatA* (*adr*) gene decreases the inherent minimum inhibitory concentration of penicillin required to kill *Streptococcus pneumoniae* and increases sensitivity of both this human pathogen and *Staphylococcus aureus* to the lysozymes of host immune systems. OatA is predicted to be a bi-modular protein that contains an N-terminal transmembrane domain and a C-terminal extracellular catalytic domain. We have cloned *oatA* from both *S. pneumoniae* and *S. aureus* coding for its C-terminal catalytic domain (OatA_c) in frame with an N-terminal His₆-tag. Expression conditions were established for the overproduction of large quantities of soluble proteins which have been purified to apparent homogeneity by a combination of affinity and ion-exchange chromatographies. Both enzymes were demonstrated to function as O-acetyltransferases using the pseudosubstrate acetyl-donor *p*-nitrophenylacetate and chitooligosaccharide acceptors. Kinetic analyses indicated the transferases have specificities for polysaccharides of increasing length and ESI-MS/MS analyses of reaction products suggests they prefer to modify terminal non-reducing residues. The three-dimensional structure of OatA_c has been solved to 1.1 Å resolution and it is found to have similarity to the SGNH superfamily of hydrolases adopting an α/β hydrolase-like fold. The invariant Asp568, His571 and Ser438 residues are aligned in a shallow pocket on the protein's surface and their respective replacement with Ala confirmed their participation in the catalytic mechanism of the enzyme. Thus, like the PatB paralog of Gram-negative bacteria, OatA is proposed to use a double-displacement mechanism of action similar to that of the serine esterase superfamily of enzymes; however, in the acetyltransfer mechanism water is excluded from the active site and replaced with the C-6 hydroxyl group of the acceptor saccharide residue in PG. Our elucidation of the catalytic pathway of OatA involving a catalytic triad of Ser, His and Asp residues provides valuable insight for the search for, and development of, inhibitors that may serve as leads for the generation of new classes of antibiotics.

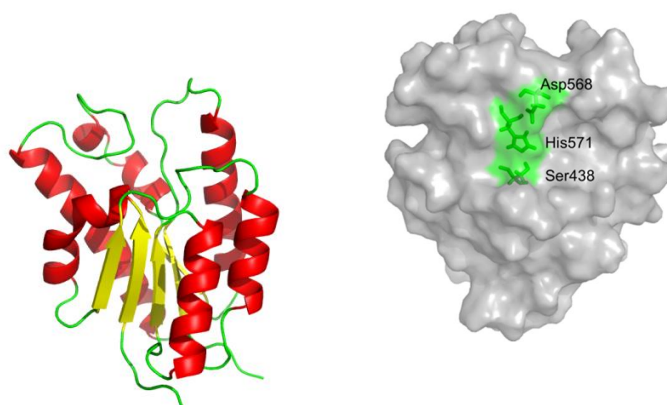


Fig 1. Structure of *S. pneumoniae* OatA

Complete switch from α 2,3- to α 2,6-regioselectivity in *Pasteurella dagmatis* β -D-galactoside sialyltransferase by active-site redesign

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α 2,3- and α 2,6-sialic acid capped oligosaccharides are of high importance for human glycobiology. Currently there is great interest in synthetically generated sialylated human milk oligosaccharides (HMOs), in sialyllactose in particular, as commercial food ingredients with a health promoting effect. Stereo- and regiocontrol are critical problems needing special attention during sialoside synthesis. For selective biocatalytic sialylation sialyltransferases are very useful catalysts that offer high regioselectivity. We present for the first time a structure-guided active-site redesign of a family GT-80 β -D-galactoside sialyltransferase (from *Pasteurella dagmatis*)^{1,2} to achieve complete switch in enzyme regioselectivity from α 2,3 in wild type to α 2,6 in a designed P7H-M117A double mutant.³ Biochemical data for sialylation of lactose and high-resolution protein crystal structures demonstrate a highly precise active-site enzyme engineering. We show the application of this unique pair of regio-complementary sialyltransferases for the synthesis of α 2,3/ α 2,6-sialyllactose and α 2,3/ α 2,6-sialyl-*N*-acetylglucosamine. Alternative 3'- or 6'-sialylation of protein asialo-*N*-glycans will also be demonstrated. In this way valuable insight into structure-function relationships of family GT-80 sialyltransferases was obtained.

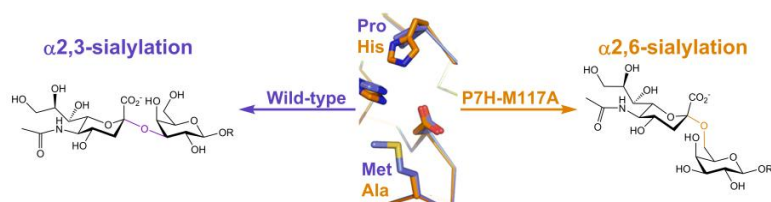


Fig 1. Structurally-guided design of a P7H-M117A double mutant of *P. dagmatis* wild-type α 2,3-sialyltransferase resulted in a completely regioselective and highly efficient α 2,6-sialyltransferase.

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Structure and function in the GH53 β -1,4-galactanase family

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β -1,4-galactanases are found in prokaryotic, eukaryotic and archaeal microorganisms, where they are thought to be involved in digestion of galactan side chains of plant pectins. They are found exclusively in the GH53 CAZY family. We have over the years determined a number of structures and studied in detail the structure-function relationships in the family. In this presentation we focus in particular on three aspects, by discussing both own unpublished results and information already available in the literature.

- 1) Substrate specificity: GH53 enzymes belonging to different domains of life show different substrate preferences and different transglycosylation abilities (1-3), which will be discussed in light of structures obtained in complex with oligosaccharides (1,4).
- 2) pH dependence of activity and stability: structures of galactanases with different pH optima are known (5) and efforts to computationally predict and understand the different pH dependences in terms of structure will be illustrated, including data on a variant with shifted pH optimum.
- 3) Stability and thermostability: structural features important for the stability of galactanases will be illustrated by comparison of structures of GH53 enzymes with different stability profiles (5), as well as mutagenesis studies aimed at the generation of thermostable variants (6-7).

Acknowledgements

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Determinants of substrate specificity in chitin oligosaccharide deacetylases: how loops define the de-*N*-acetylation pattern

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Chitin processing, mainly in the form of depolymerization and de-*N*-acetylation reactions, generates a series of derivatives including chitosan and chitooligosaccharides (COSs), which play remarkable roles in nature. COSs are particularly involved in molecular recognition events, including the modulation of cell signaling and morphogenesis, the immune response, and host-pathogen interactions. Most of the biological activities associated with COSs seem to be largely dependent on the degree of polymerization and the specific acetylation pattern, which define the charge density and the distribution of GlcNAc and GlcNH₂ moieties in chitosan and COS.

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 CE4 enzymes active on COS is diverse, some being specific for a single position, others showing multiple attack. *A major challenge is to understand how CDAs specifically define the distribution of GlcNAc and GlcNH₂ moieties in the oligomeric chain.*

To address this question, we here report a structural/functional analysis of CDAs which includes:

- a) The crystal structure of the *Vibrio cholerae* chitin oligosaccharide deacetylase (VcCDA or VcCOD) in four relevant states of its catalytic cycle [1]. Two enzyme complexes with chitobiose and chitotriose represent the first 3D structures of a CDA with its natural substrates in a productive mode for catalysis, thereby unraveling an induced-fit mechanism with a significant conformational change of a loop closing the active site.
- b) Based on these novel structures and structural analyses of CE4 enzymes active on COS, we propose a “Subsite Capping Model” in which the deacetylation pattern exhibited by different CDAs is governed by critical dynamic loops that shape and differentially block accessible subsites in the binding cleft of CE4 enzymes. The model provides the bases for the rational design of CDA enzymes.
- c) Engineered variants to introduce novel deacetylation specificities.

This work is supported by EU grants ChitoBioEngineering and Nano3Bio aimed at the biotechnological production of novel chitosan oligo- and polysaccharides for industrial and medical applications. (<http://www.nano3bio.eu>)

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Molecular basis for the epimerization of oligosaccharides by cellobiose 2-epimerase

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Cellobiose 2-epimerase (CE), which has been first found in a ruminal bacterium, *Ruminococcus albus*, catalyzes the epimerization of 2-OH group of reducing end sugar residue of β 1-4 linked oligosaccharides including cellobiose, lactose, and β 1-4 mannosiose. Functional analysis of CE-like proteins revealed that CE is distributed in various bacteria including *Bacteroides fragilis* and *Rhodothermus marinus*. In the genome of *B. fragilis*, the CE gene is a member of a gene cluster including the genes encoding β -mannanase and 4-*O*- β -D-mannosyl-D-glucose phosphorylase. Thus CE is predicted to convert β 1-4 mannosiose to 4-*O*- β -D-mannosyl-D-glucose for further phosphorolysis in the metabolism of β -mannan. As epilactose (4-*O*- β -D-galactosyl-D-mannose), produced from lactose by CE, has a prebiotic property, CE is an attractive enzyme for production of a functional foodstuff.

In this study, we have determined the crystal structures of CEs from *R. albus* [1] and *R. marinus* [2]. In *R. marinus* CE (*RmCE*), structures in complex with 4-*O*- β -D-glucosyl-D-mannose, epilactose, and cellobiitol (a competitive inhibitor. K_i , 6.13 mM) were determined. The overall folds of these CEs were $(\alpha/\alpha)_6$ barrel, similar to those of *N*-acetylglucosamine 2-epimerase (AGE) and aldose ketose isomerase (AKI). In the CEs, two catalytic His residues and amino acid residues surrounding the reducing end sugar part are situated at the positions corresponding to those of AGE and AKI, suggesting that these enzymes share a catalytic mechanism. In the complex of *RmCE* and cellobiitol, cellobiitol took *cis*-enediol like conformation, and C1-C2 bond of the D-glucitol part of cellobiitol was positioned between catalytic His259 and His390. The H2 proton of the D-glucitol part was close to His390, and His390 is the most feasible candidate to abstract the H2 proton from D-glucose residue at the reducing end of substrate. His259 acts as a general acid catalyst to donate a proton to the C2 position of *cis*-enediol intermediate, and the D-glucose residue is converted to D-mannose residue. In contrast to AGE and AKI, which act on monosaccharide substrates, CE has very low activity to a monosaccharide. The k_{cat}/K_m of *RmCE* for D-mannose was 3,670-fold lower than that of β 1-4 mannosiose. Structural analysis showed that Trp385 and Asp188 of *RmCE* have stacking and hydrogen bonding interactions with sugar part at the non-reducing end of substrate, respectively. W385A and D188A mutations more severely decreased activity to disaccharide substrates than to D-mannose, and thus these residues contribute to the disaccharide-selectivity of CE.

Acknowledgements

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Sugar oxidoreductions at the crossroads of mechanistic enzymology and biotechnological application

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Simple (single-step) and complex oxidoreductive transformations are widely used in biology to assimilate sugars into cellular metabolism and to generate building blocks for biosynthesis. In biotechnology sugar oxidoreductases have important applications in synthetic bioconversions via catalytic cascade or whole-cell systems. The example of yeast xylose fermentation is used to demonstrate structure- and mechanism-based engineering of sugar oxidoreductases for integration into a recombinant metabolic pathway for xylose-to-ethanol conversion optimized for product yield and fluxional efficiency.^{1,2} Enzymes of the biosynthesis of UDP-glucuronic acid,³ UDP-xylose^{4,5} and UDP-apiiose are shown to catalyze multi-step oxidoreductive reactions at their respective active site. Principles used in catalysis are described based on evidence from protein structures and mechanistic studies.

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Functional characterization of a set of fungal lytic polysaccharide monoxygenase secreted by *Podospora anserina*

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Lignocellulosic biomass is the largest renewable source of carbohydrates for the production of biofuels, biomaterials, and high-value products. The main component of plant cell wall is cellulose, a linear polymer of β -1-4 linked glucose units arranged in linear microfibrils that form very recalcitrant crystalline-like structures. The understanding of enzymatic polysaccharide degradation has progressed intensely in the past few years with the identification of a new class of fungal secreted enzymes, the lytic polysaccharide monoxygenases (LPMOs) that enhance cellulose conversion. The family AA9 comprises about 300 fungal members widely distributed in the genomes of most ascomycetes and basidiomycetes. A striking feature is the extreme expansion in genes encoding AA9s observed in the biomass degrader *Podospora anserina* (i.e. 33 genes) that represents an interesting model to study the oxidative deconstruction of lignocellulose. In this study, we investigated a set of AA9 LPMOs identified in the secretomes of *P. anserina* (Poidevin et al., 2014). Six AA9 LPMOs were expressed heterologously using the methylotrophic yeast *Pichia pastoris*. LPMO activity was assayed on cellulose in synergy with the cellobiose dehydrogenase from the same organism. We showed that the total release of non-oxidized and oxidized oligosaccharides from cellulose was promoted by the presence of a carbohydrate-binding module from the family 1. The investigation of their regioselective mode of action using ionic chromatography and mass spectrometry revealed differences in the position of the oxidative cleavage of cellulose (at the C1 and/or C4 position). This study provides novel insights into the mode of cleavage and substrate specificities of fungal AA9 LPMOs that will facilitate their application for the development of future biorefineries.

Acknowledgements

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Glucooligosaccharide oxidases: determinants of activity and use in carbohydrate modification

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Enzymatic oxidation of polysaccharides can alter the rheology of corresponding polymers and facilitate regioselective modification of complex carbohydrates. Moreover, since enzyme catalyzed reactions can be performed in relatively mild reaction conditions, enzymatically modified polysaccharides generally retain the degree of polymerization and crystallinity of the starting substrate. This presentation will describe our characterization and application of a glucooligosaccharide oxidase from *Sarocladium strictum* (namely, GOOX). GOOX is classified by the CAZy database as a family 7 auxiliary activity, and like other flavin carbohydrate oxidases, targets the hydroxyl group of the anomeric carbon. The comparatively accessible substrate binding site of GOOX enables oxidation of oligomeric and polymeric substrates, while activity on monosaccharides is comparatively low. Catalytic efficiencies on cello-oligosaccharides were similar to those measured using xylo-oligosaccharides with or without arabinose substituents at positions C3 and/or C2 of the non-reducing xylose. By contrast, the presence of uronic acid substituents decreased the catalytic efficiency of GOOX, largely through increasing K_m values. Mutagenesis studies have identified substrate binding subsites near the substrate binding pocket of GOOX similar to paradigms observed for many glycoside hydrolases; eight additional amino acid substitutions were introduced in an effort to identify determinants of substrate selectivity. Most notably, the W351A substitution at a substrate binding subsite increased k_{cat} values up to 3-fold on cello- and xylo-oligosaccharides while alleviating substrate inhibition. In addition to oxidizing oligosaccharides, GOOX activity was confirmed using a range of plant polysaccharides. Inspired by the precedence to enhance cellulase performance on insoluble polysaccharides through fusion to cellulose-binding modules, we investigated the impact of carbohydrate-binding modules from different CBM families, on GOOX activity towards high molecular weight and insoluble polysaccharides. In line with trends observed for hydrolytic enzymes, greatest benefits of CBM fusion were observed when using low concentrations of insoluble polysaccharides. Finally, GOOX alone and in combination with other carbohydrate oxidases is being developed for applications in biosensing and valorization of hemicellulose. Recent results towards these ends will be described.

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Engineering of pyranose oxidoreductases for bio-fuelcell applications

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Pyranose dehydrogenase (PDH) and pyranose 2-oxidase (POx) are flavoproteins that both catalyze the oxidation of free, non-phosphorylated sugars to the corresponding ketosugars. Pyranose dehydrogenase is limited to a narrow range of electron acceptors and reacts extremely slowly with dioxygen, whereas pyranose 2-oxidase displays high oxidase as well as dehydrogenase activity. For bio-fuelcells, oxygen reactivity is undesirable as it leads to electron "leakage" and the formation of damaging hydrogen peroxide; for biocatalytic applications, oxygen reactivity can be advantageous, as oxygen is ubiquitous and freely available. Site-saturation mutagenesis libraries of twelve (PDH) and eleven (POx) amino acids around the active sites were expressed and screened for oxidase and dehydrogenase activities. Only one PDH variant displayed increased oxygen reactivity to a minor degree. Histidine 103, carrying the covalently attached FAD cofactor, was substituted by tyrosine, phenylalanine, tryptophan and methionine. Variant H103Y displayed a five-fold increase of oxygen reactivity. Stopped flow analysis revealed that the mutation slowed down the reductive half-reaction whereas the oxidative half-reaction was affected to a minor degree. No alterations in the secondary structure were observed. Disruption of the FAD bond also had negative effects on thermal and conformational stability. In POx, variants T166R, Q448H, L545C, L547R and N593C displayed significantly reduced oxidase activities (between 40% and 0.2% of the wildtype) concomitant with unaffected or even increased dehydrogenase activity, dependent on the electron acceptor used (DCPIP, 1,4-benzoquinone or ferricenium ion). Kinetic characterization showed that both affinity and turnover numbers can be affected. The switch from oxidase to dehydrogenase activity was also observed electrochemically, and the effect of the mutations was rationalized based on structural properties. Additionally, we engineered PDH by removing several of the enzyme's N-glycosylation sites, in order to improve electron transfer by reducing the distance of the active site to the electron surface and facilitate denser enzyme packing. We found improved properties including a low level of Direct Electron Transfer with a variant lacking two glycosylation sites close to the active site, with only small negative effects on the variant's expressability and stability, and determined that the bulk of the overglycosylation of the recombinantly expressed enzyme is located on only one glycosylation site.

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The role of carbon starvation in the induction of enzymes that degrade plant-derived carbohydrates in *Aspergillus niger*

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The saccharification of lignocellulosic biomass for the production of second generation biofuels requires cheaper and more effective enzyme mixtures. Fungi are an important source of such enzymes, but the understanding of the regulation and induction of the encoding genes is still incomplete. To explore the induction mechanism, we analysed the response of the industrially important fungus *Aspergillus niger* to wheat straw, with a focus on events occurring shortly after exposure to the substrate. RNA sequencing showed that the complexity of transcriptional response increased over time.

Importantly, the influence of carbon starvation during lignocellulose degradation was demonstrated by a substantial overlap in CAZyme-encoding transcripts induced during both early carbon starvation and early exposure to straw. The up-regulation of the expression of a high number of genes encoding CAZymes that are active on plant-derived carbohydrates during early carbon starvation suggests that these enzymes could be involved in a scouting role during starvation, releasing inducing sugars from complex plant polysaccharides. We show that carbon-starved cultures indeed release CAZymes with predicted activity on plant polysaccharides. Analysis of the enzymatic activity and the reaction products, indicates that these proteins are enzymes that can degrade various plant polysaccharides to generate both known, as well as potentially new, inducers of CAZymes¹

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Esterases of *Myceliophthora thermophila* C1 help in the degradation and modification of lignocellulosic material

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Lignocellulosic biomass is the most abundant renewable resource on Earth. Its value is based on the carbohydrate polymers that are present in plant cell walls. Here, depending on the type of plant, one finds a cellulose-hemicellulose network embedded in a matrix of pectic or lignin substances. The strength of the network is improved by ester bonds of cinnamic acids such as ferulic acid that cross-link hemicelluloses with lignin or with pectin, hindering the enzymatic degradation of the whole system. Besides ferulic acid esters, also acetic acid esters and glucuronic acid esters can be present in hemicelluloses and crosslink with other cell wall components (glucuronic acid). In the degradation of lignocellulosic materials, a key step in opening up this tight structure is the removal of the esters.

Dyadic develops the filamentous fungus *Myceliophthora thermophila* C1 as a proprietary protein production platform for the efficient production of tailor-made enzyme mixtures for the degradation or modification of lignocellulosic biomass. This fungal strain encodes, along with many cellulases and hemicellulases, various accessory enzymes including eight feruloyl, thirteen acetyl and two glucuronoyl esterases. Five of the feruloyl esterases, six of the acetyl esterases (classified as CE1, CE3, CE4, CE5 and CE16) and two glucuronoyl esterases (CE15) found in the C1 genome have been produced individually in a dedicated C1 host strain, and subsequently purified and characterized. Results show that they all have differences in their mode of action and substrate specificities, even if they belong to the same enzyme family. These differences in mode of action and substrate specificity form the basis for the use of these enzymes in a variety of applications, for instance in processes to make bio-based fuels and chemicals processes.

Processive action of *Rasamsonia emersonii* cellobiohydrolase Cel7A

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Rasamsonia emersonii cellobiohydrolase *ReCel7A*, which was formerly called *Talaromyces emersonii* Cel7A, is a thermostable cellulase that occurs naturally without a carbohydrate-binding module (CBM). The three-dimensional structure of the enzyme has been solved (Grassick et al., 2004). Due to its thermostability (Voutilainen et al., 2010; Voutilainen et al., 2013) and good lignin-tolerance (Rahikainen et al., 2013), *ReCel7A* is a highly relevant enzyme for total hydrolysis applications for the biofuels industry.

In this study, *ReCel7A* enzyme with and without a family-1 CBM was studied using various crystalline and amorphous cellulosic substrates. In addition, the action of *ReCel7A* was monitored with the high-speed atomic force microscopy (HS-AFM) that allows real-time observation of cellobiohydrolase movement on crystalline cellulose surfaces (Igarashi et al., 2011). The paper discusses the processivity, as well as the differences in mode of action between the widely studied *Trichoderma reesei* Cel7A and *ReCel7A*.

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Hydrolysis of arabinoxylo-oligosaccharides and wheat flour arabinoxylan by α -L-arabinofuranosidases

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Arabinofuranosidases which hydrolyse terminal α -L-arabinofuranose from polymeric arabinoxylans are termed arabinoxylan arabinofuranosidases (AXH) and these have been divided into two groups depending on their substrate specificities. The enzyme AXH-m acts on α -L-(1-2)- and (1-3)-linked Araf units on monosubstituted Xylp residues¹, whereas AXH-d3 releases only α -L-(1-3)-linked Araf units from disubstituted Xylp residues² within arabinoxylan. Relatively little is known on the action of specific α -L-arabinofuranosidase on defined oligosaccharides. In one recent study, Borsenberger *et al.*³ reported on the action of several α -L-arabinofuranosidases from *B. adolescentis* and *Thermobacillus xylanilyticus* on A²⁺³XX and three synthetic di-arabinofuranosylated substrates and demonstrated that these substrates can be used to characterize and probe selectivity of the arabinoxylan-active α -L-arabinofuranosidases. Work on specific native AXOS has been limited by the lack of availability of these substrates. In this paper we report on methods for the preparation of specific AXOS, with both doubly substituted D-Xylp residues and with singly substituted D-Xylp residues (1,3- and 1,2-linked and either terminal or within the 1,4- β -D-xylo-oligosaccharide backbone), including A³X, A²XX, A³XX, XA³XX, XA²XX and A²⁺³XX. Relative rates of hydrolysis of these oligosaccharides by several α -L-arabinofuranosidases are shown in Table 1. Clearly there are differences in the rates of hydrolysis of AXOS by the different enzymes, even differences between the AXH-m. These oligosaccharides should prove valuable in defining the substrate binding requirements and specificities of the wide spectrum of α -L-arabinofuranosidases under investigation.

Table 1. Relative rates of hydrolysis of arabinoxylo-oligosaccharides by α -L-arabinofuranosidase enzymes.

Substrate	<i>B. adolescentis</i> Q5JB56 (GH43)	<i>B. ovatus</i> BACOVA_03417 A7LZZ1 (GH43)	<i>B. ovatus</i> BACOVA_03421 A7LZZ4 (GH43)	<i>B. ovatus</i> BACOVA_03425 A7LZZ8 (GH43)	<i>U. maydis</i> O4P6F4 (GH62)	<i>A. niger</i> B3GQR2 (GH51)
A ³ X*	0	0	2.6	1.9	0.6	133
A ² XX*	0	<0.02	28.5	24.9	2.7	21
XA ² XX*	0	0	28.5	10.2	6.3	5
XA ² XX and XA ³ XX*	0	0	28.5	27.7	26.4	5
A ²⁺³ XX*	285	208	0	0	0	4
Arabinoxylan (wheat) **	67	650	16	23	30	0.7
Arabinan (sugar beet) **	0.54	0.53	-	0.013	0.003	10.3
Debranched Arabinan (sugar beet) **	0	0	-	0	0	0.31
p-NP- α -L-Araf***	0.031	0.325	-	0.328	0.012	29

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Exploring plant cell wall xylan biosynthesis, structure and function

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The two most abundant polysaccharides in plant cell walls are cellulose and xylan. Interactions between these polysaccharides are important for cell wall properties, but the nature of the interactions are not understood. To discover enzymes of cellulose and xylan biosynthesis we generated a catalogue of plant Golgi proteins using a quantitative proteomics approach. Glycosyltransferases of unknown function form a substantial proportion of proteins in the plant Golgi, and a substantial proportion are not yet catalogued in CAZy GT families (1). Using mutant *Arabidopsis* as a model system, we have found enzymes involved in xylan backbone synthesis and substitution with sugars. Surprisingly, digestion of the xylan polysaccharide revealed that the spacing of sugar and acetate decorations follows a precise periodic pattern. We propose this spacing allows some of the cell wall xylan to fold as a flat two-fold helical screw ribbon in order to hydrogen bond with the glucan chains in cellulose microfibrils (2). Multidimensional ¹³C solid state NMR of intact plant cell walls suggests that xylan indeed is present in multiple conformations (3). We propose a model for the assembly and molecular architecture of the xylan:cellulose interactions in plant cell walls.

Acknowledgements

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Understanding the effect of overexpression of fungal acetyl xylan esterase (AXE1) in hybrid aspen

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Xylan is one of the most abundant biopolymers on the earth. 60-70% of xylopyranosyl residues in hardwood xylan backbone are acetylated at *O*-2 and/or *O*-3 position. Enzymatic and chemical deacetylation of xylan helps in increasing the saccharification potential of lignocellulosic biomass. Hence, we would like to study the feasibility of *in planta* xylan deacetylation for improved saccharification properties of wood.

Xylan acetyl esterase AXE1 from *Aspergillus niger* was expressed in hybrid aspen and targeted to cell walls. The independent transgenic lines showed decreased in xylan acetylation. Their growth was not affected. The overall composition of cell wall was unchanged but crystallinity of cellulose was increased. Interestingly, the S/G ratio was decreased in the lignin of transgenic lines. To understand the source of this difference, we isolated and we are currently analyzing different fractions of lignin carbohydrate complexes (LCC). Enzymatic saccharification of transgenic lines yielded more glucose without pretreatment and after acid pretreatment as compared to wild type. The results indicate that post synthetic modification in *O*-acetylation of xylan is a promising approach to increase biofuel production and to study complex interactions between different cell wall polymers.

Bioinspired model assemblies of plant cell walls as sensors for unravelling interaction features of CAZymes

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Carbohydrate-active enzymes (CAZymes) play a central role in the deconstruction of plant cell wall biomass. But their activity is often restricted by sterical hindrances that limit their accessibility to substrate and by non-specific interactions that stick enzymes far from their substrate. Generally, the biochemical characterization of CAZymes is performed with pure isolated polymers or synthetic molecules, which do not reflect the chemical and structural complexity of plant cell walls. Thus there is a need to better assess the interactions of CAZymes with relevant materials.

To achieve this purpose, we have first designed bioinspired model assemblies that contain some of the polymers and covalent interactions found in plant cell walls. These assemblies contain feruloylated arabinoxylans (FAXs), cellulose nano-crystals (CNCs) and model lignin (DHP) at various concentrations in solution (free polymers) or in gels (cross-linked polymers) [1]. Then, starting from a set of modulated endoglucanases belonging to glycoside hydrolase family 45, appended or not to one CBM or five CBMs from family 1 [2], we have grafted these proteins to a fluorophore so that they become fluorescent probes. Each probe has been directly embedded into the different assemblies and their mobility and interactions evaluated by confocal microscopy using the FRAP technique [3].

Overall, results show that the different controlled features related to the assemblies (solution or gel, polymer concentration, water content) and to the probes (type, size) have different impact on affinity, varying in this order: probe size > DHP concentration > FAX concentration. More importantly, the GH45 endoglucanase interacts with DHP through some surface motifs that are partially masked when the enzyme is appended to at least one CBM, making the enzyme alone more sensitive to interactions with lignin. Bioinspired model assemblies are therefore relevant for identifying and hierarchizing features influencing CAZymes affinity that restrict their mobility, thus hampering their activity.

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Discovery of original α -transglucosylases from *Leuconostoc citreum* NRRL B-1299 and NRRL B-742 for the synthesis of tailor-made α -glucans

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Due to their renewability, biodegradability and carbon-neutrality, bio-sourced polymers represent attractive alternatives to polymers derived from carbon fossil fuels, as revealed by their broad range of applications in food & feed, agriculture, health, or in chemical industries. In this context, the α -transglucosylases produced by some lactic acid bacteria are of particular interest as they catalyze, from sucrose, the synthesis of high molar mass α -glucans, glucooligosaccharides or gluco-conjugates which offer access to a large panel of products varying in term of size and linkage specificity¹. These α -transglucosylases are classified in GH70 family, which comprises today 258 sequences for only 59 enzymes biochemically characterized². To broaden GH70 applications, deepen structure function relationship studies and identify determinants of linkage and product specificity, a genomic approach focused on two original strains of *L. citreum* sp. was initiated. The two selected strains, *L. citreum* NRRL B-1299 and NRRL B-742, were identified in the fifties as producers of original α -glucans highly branched with α -(1–2) or α -(1–3) linked glucosyl units, respectively. However, strong association to the cell wall of the enzymes involved in polymer synthesis limited their isolation and characterization.

Genome sequencing and analysis of the two strains allowed to draw up the full inventory of their GH70 encoding genes, and the discovery of a battery of GH70 enzymes never described before. Genome comparison and description of the main characteristic of two branching sucrases specialized in dextran grafting through α -(1–2) or α -(1–3) linkages, as well as two dextransucrases dedicated to low molar mass dextrans synthesis will be presented and discussed with regard to their specificity and their potential for technical innovation.

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Marine-derived bacterial polysaccharides are valuable sources of glycosaminoglycans

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The bacteria from marine ecosystems are a highly valuable resource for original biomolecules and biocatalysts. In particular, marine prokaryotes offer a source of safe, biocompatible, biodegradable and renewable products especially polysaccharides. Based on their structural features, specific biological functions have been highlighted; they are of great interest in human health as glycosaminoglycans-like (GAG-like) molecules. The biological activity of glycopolymers derives from their molecular structure including molecular size as well as sulphation pattern. The use of polysaccharides, in particular for human health, requires the control of molecular characteristics and the understanding of the biosynthesis mechanism and regulation can help in controlling the final molecular features. On the other way, to maximize polysaccharide applications as GAG-like molecules and generate new biological functions, these molecules may be suitably chemically or enzymatically engineered (depolymerised and sulphated). The characteristics of enzymatic methods to get oligosaccharides of biological relevance meet well the needs of better control of the modification process and of environmentally safer processing steps. Therefore, new specific carbohydrate active enzymes and sulfotransferases are needed. The bacteria that produce polysaccharides are also a source of key enzymes for the production of tailor-made polysaccharides.

The study of microbial life adapted to deep-sea hydrothermal vents conditions is a way of discovering new biomolecules with innovative properties and potential applications in human health. Marine bacteria able to produce exopolysaccharides having glycosaminoglycan properties belong to *Vibrio* and *Alteromonas* genera; in particular, some of them are naturally sulfated; this is very rare within the microbial world. Data on three exopolysaccharide (EPS) producing bacteria –*Vibrio diabolicus*, *Alteromonas infernus*, and HYD721 strain– isolated from deep sea habitats will be presented. These would include EPS production in bioreactors, molecular mechanisms of the polysaccharide biosynthesis, comparative genomic and phenotypic features as well as data on carbohydrate active enzymes especially sulfotransferases.

These studies provide a better basic knowledge on the biosynthesis of bioactive polysaccharides and would provide means to control the production as well to engineer it.

Spider silk mimicking assembly of nanocellulose

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Natural materials are known for their strength and toughness. Spider silk and cellulose are both nature's high end fibres having good mechanical properties in the range of man-made high-tech materials^{1,2}. Composites made from nanocellulose are attractive for material applications due to the strength but also the low cost and biocompatibility of the raw material. Spider dragline silk produced by orb-weaving spider is a unique fibrous protein material consisting of three clearly different sequence sections: 1) core of the spidroin is highly repetitive sequence of polyalanine stretches interrupted with glycine-rich regions, 2) in the ends of the repetitive core, the spidroin has globular modules called N-terminal module and 3) C-terminal module. Both N- and C- terminal modules have a role when spidroin protein monomers are being assembled to form silk fibres. The N-terminal module forms a homodimer and firmly interconnects the spidroins when the pH in the spider silk gland is lowered from pH 7 to pH 6 – 5.5³.

The excellent mechanical properties of both spider silk and cellulose are due to the precise nanoscale organisation of the molecules in nature, which is a grand challenge when nanocellulose or silk materials are produced. We utilized a carbohydrate binding module (CBM) to attach the spider silk N-terminal module on cellulose nanofibers for mimicking nature's way to assemble spider silk spidroin as functional fibres. The biochemical properties of the recombinant fusion proteins were studied and it became clear that the pH dependent dimerization of NT-module is functional in the fusion proteins and not affected by the CBM fusion. In addition, the binding properties of the fusion proteins on nanocellulose were studied and the rheological properties of the nanocellulose/protein mixture revealed clear self-organization of the material when the pH was changed to favour the dimerization of the NT-module.

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Multiple CBMs enhance starch degradation by members of the human gut microbiota

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Starch is an abundant carbohydrate in our diet and human gut bacteria have evolved many different strategies for scavenging this polysaccharide. We have studied the molecular mechanism of starch degradation and import by two representative members of the major gut bacterial phyla, the Gram-negative Bacteroidetes and the Gram-positive Firmicutes. In both phyla starch is targeted to the surface of the cell but processed by distinctly different amylases and import machinery. The starch utilization system (Sus) of *Bacteroides thetaiotaomicron* is comprised of five cell surface proteins that work together to bind, degrade and import starch. The lipoproteins SusDEFG have a combined total of eight distinct non-catalytic starch-binding sites that have apparently distinct roles in starch sensing and uptake. Among these, SusD, SusE and SusF have unique structures that facilitate the capture and import of maltooligosaccharides [1, 2]. SusG, a surface amylase, features a starch-binding CBM58, inserted within the catalytic domain, but is dispensable in starch utilization[3]. Our single molecule imaging of SusG in live *B. theta* suggests that this protein interacts dynamically with other Sus proteins during starch digestion[4]. In contrast to the Sus employed by members of the Bacteroidetes, members of the Firmicutes, such as *Eubacterium rectale*, encode one or more cell-wall anchored amylases with multiple tandem starch-binding domains. Our recent work with *E. rectale* has demonstrated its ability to digest granular cornstarch to maltotetraose that is then scavenged by two abundant maltooligosaccharide-binding proteins as part of ABC transporters that are specific for maltotriose and larger oligosaccharides[5]. Comparative genomic analysis of this pathway suggests that it is highly conserved among related Firmicutes, and may account for the ability of these bacteria to flourish in individuals that consume a diet high in resistant starch. Through a detailed understanding of how human gut bacteria acquire carbohydrate nutrition in the highly competitive gut ecosystem, we can develop prebiotic and probiotic strategies to manipulate the composition of this community towards improved human health.

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Functionality of granule-bound starch synthase from the waxy barley cultivar CDC Alamo

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Amylose synthesis in plants is strictly dependent on activity of Granule-Bound Starch Synthase (GBSS) enzyme types. *Near-waxy* or *waxy* cultivars are starch crops containing either little or no amylose. Such cultivars have been identified among maize, potato, wheat, barley and rice. This phenotype has so far been associated with a single locus which has been mapped to GBSS-type genes. Most such waxy varieties are a result of either low or no expression of a single GBSS gene. But there are waxy varieties, where the GBSS enzymes are expressed at a normal level. For such types, non-silent SNPs have been suggested to result in loss of catalytic activity. We here cloned the cDNA and characterized the HvGBSS1a enzyme from the amylose-free barley variety CDC Alamo, and found that the HvGBSS1a from CDC Alamo actively synthesizes amylose both in vitro and in vivo. GFP tagged HvGBSS1a's of both the non-waxy type and the CDC Alamo type were found to localize in concentric circles strictly within starch granules in transgenic barley lines. Finally HvGBSS1a from CDC Alamo was fully able to complement the waxy phenotype when expressed transgenically in other waxy varieties.

In conclusion these observations are not in agreement with HvGBSS1a from the amylose-free barley variety CDC Alamo being catalytically inactive, suggesting that the amylose-free phenotype of CDC Alamo is related to other factors than GBSS.

Glucan phosphatases utilize different mechanisms to bind starch and glycogen

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Lafora disease (LD), a fatal neurodegenerative epilepsy, and starch metabolism are linked by a family of enzymes that we discovered called glucan phosphatases. Glucans are the most abundant polymer in plants, with cellulose serving as the structural component and starch as the energy reserve. Instead of starch, humans utilize glycogen as their primary carbohydrate storage molecule. Recent work shows that the metabolism of both starch and glycogen is dependent on the action of glucan phosphatases.

Plants release the energy in starch via a three-step process: starch phosphorylation, degradation, and dephosphorylation. Dikinases phosphorylate outer starch glucose units to make them water-soluble and enzyme accessible so that amylases can release maltose and glucose. Following amylase activity, the phosphate must be removed by glucan phosphatases. In the absence of glucan phosphatases, plants cannot access the energy stored in starch and the starch granules grow in size while plant growth is stunted.

The *EPM2A* gene encodes laforin and recessive mutations in *EPM2A* result in LD. We demonstrated that laforin is a human glucan phosphatase. In the absence of laforin activity, glycogen transforms into a hyper-phosphorylated, water-insoluble, starch-like Lafora body (LB). LBs are the suspected cause of neuronal apoptosis, neurodegeneration, and eventual death of LD patients.

We recently determined crystal structures of the *Arabidopsis* starch phosphatase Starch Excess 4 (SEX4) with phospho-glucan bound at 1.62Å. In addition, we determined the structure of *Like Sex Four2* (LSF2) both with and without phospho-glucan product bound at 2.3Å and 1.65Å, respectively. Most recently, we determined the structure of human laforin bound to phosphoglucan product. Our data demonstrate that each phosphatase utilizes a unique mechanism to bind and dephosphorylate its substrate.

These structures are the first of glucan phosphatases, and they provide new insights into the molecular basis of this medically-, agriculturally-, and industry-relevant enzyme family as well as their unique mechanisms of catalysis, substrate specificity, and interaction with glucans.

Secondary structure reshuffling modulates the enzymatic activity of a GT-B glycosyltransferase at the membrane interface

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The phosphatidylinositol mannosyltransferase PimA is an essential membrane-associated enzyme that initiates the biosynthetic pathway of key structural elements and virulence factors of *Mycobacterium tuberculosis*, such as phosphatidylinositol mannosides (PIM), lipomannan and lipoarabinomannan (1, 2, 3). PimA is an amphitropic glycosyltransferase that belongs to the GT-B superfamily, composed of two Rossmann-fold domains separated by a central cleft that includes the catalytic center (4). As observed for other GT-B enzymes, binding of the sugar donor substrate GDP-Man to PimA stabilizes a ‘closed’ state, in which crucial residues from the N- and C-terminal domains are brought together to form a functionally competent active site. In contrast, binding of the sugar acceptor substrate phosphatidylinositol (PI) was observed to have a destabilizing effect on PimA, possibly due to the formation of a more relaxed enzyme-ligand complex (5). The structural flexibility of PimA was further stressed by recent single-molecule and small-angle X-ray scattering studies, which demonstrated that PimA displays a highly dynamic N-terminal domain (6).

To advance our understanding of the molecular mechanisms that govern substrate binding and catalysis, we have now determined the crystal structure of the apo form of PimA. The most striking feature of this crystal form is that apo PimA did not exhibit the ‘classical’ Rossmann-fold topology, which was previously observed for the same enzyme in complex with GDP or GDP-Man. We demonstrate that the occurrence of a conformational switch between the apo and GDP-bound crystal structures of PimA must involve both β -strand-to- α -helix and α -helix-to- β -strand transitions. These structural changes seem to modulate catalysis and are promoted by interactions of the protein with anionic phospholipids in the membrane. Although scant structural information is currently available on protein catalysis at the lipid-water interface, our studies demonstrate that protein-membrane interactions might entail unanticipated structural changes in otherwise well conserved protein architectures, and suggests that similar changes may also play a functional role in other membrane-associated GT-B enzymes (7).

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Degrading sulfated sugars from the sea: novel insights into the evolution, dimerization plasticity and catalytic mechanism of the GH117s

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Marine polysaccharides differ from their terrestrial counterparts, notably, all marine algae produce sulfated polysaccharides, which are absent in land plants. The sulfated galactan agar is a key component of some red algal cell walls. Red algae synthesize an unusual carbohydrate moiety from the galactose scaffold, the α -1,3-linked bicyclic 3,6-anhydrogalactose. The synthesis of 3,6-anhydrogalactose is unique to red macroalgae and contributes to flexibility and hydration, which is important given the extreme environmental forces exerted by the ocean on the organism.

Red macroalgae are important primary producers to the marine ecosystem and a large proportion of their organic biomass is recycled through the food chain. Microbes have developed complex systems designed for the degradation of agar and other marine polysaccharides. The marine bacterium *Zobellia galactanivorans*, which was isolated from a red alga, produces five paralogous family 117 glycoside hydrolase enzymes (GH117s). The GH117s are involved in the final step of agarose degradation which results in the production of a monosaccharide. To date, there are only structure function studies on the Clade A GH117s which demonstrate exo- α -1,3-(3,6-anhydro)-L-galactosidase activity. The Clade C enzymes are predicted to have different substrate specificities. Thus, two Clade C GH117s from *Z. galactanivorans* were chosen for in-depth structural and functional analyses.

An interesting feature of the GH117s is that they form structural homodimers whereby the two monomers participate in domain swapping. The two Clade C GH117s studied exhibit different modes of dimerization as well as active site differences. Crystal soaks with oligosaccharide substrate resulted in a product complex with the β -3,6-anhydro-L-galactose moiety. The β -anomer of 3,6-anhydro-L-galactose has been hypothesized to exist but this is the first time that the structure of this unusual bicyclic sugar has been clearly elucidated. The product complex provides strong crystallographic evidence for an inverting catalytic mechanism in this family as well as insight into the structure of the unique sugar produced by hydrolysis of agarose.

Many new marine bacterial genomes containing GH117 enzymes have been released since the first phylogenetic tree was published in 2011. New phylogenetic analysis reveals novel clades within the family and suggests members from other clades may themselves have distinctive specificities. Thus, there remains much to be understood about this GH family designed for the final step in the degradation of sulfated marine galactans.

Functional metagenomics reveals novel pathways of mannoside metabolization by human gut bacteria

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The human gut hosts a complex bacterial community that plays a major role in nutrition and in maintaining human health. To face the huge structural diversity of carbohydrates which constitute their main carbon sources, intestinal bacteria produce a full repertoire of CAZymes, of which glycoside hydrolases are the main constituents. However, other types of enzymes, like glycoside-phosphorylases, participate in the breakdown of complex carbohydrates, by working in synergy with glycoside hydrolases. The prevalence of glycoside-phosphorylases and their role in carbohydrate metabolism is therefore difficult to evaluate on the basis of sequence data alone, because of their structural and mechanistic similarities with glycoside hydrolases and glycosyltransferases.

Recently, integration of biochemical, metagenomic and genomic data at the level of the entire human gut ecosystem, revealed novel mechanisms of plant and host glycan metabolization, involving mannoside-phosphorylases of different GH130 subfamilies^{1,2}. Analysis of the first crystal structure of a N-glycan phosphorylase highlighted the molecular determinants of GH130 functional specificities. Thanks to these data, these fascinating enzymes now appear as new targets to study interactions between host, dietary constituents and gut microbes, in particular in the context of inflammatory bowel diseases.

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Structural basis for arabinoxylo-oligosaccharide capture by probiotic bifidobacteria

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Glycan utilization is an important factor modulating the composition of the gut microbiota,¹ but insight into oligosaccharide uptake by this microbial community is scarce. Arabinoxylan is abundant in human diet, and its arabinoxylo-oligosaccharide (AXOS) oligomers are selectively fermented by gut adapted health stimulating bifidobacteria in the lower part of the gastrointestinal tract. The AXOS utilization locus in the probiotic bacterium *Bifidobacterium animalis* subsp. *lactis* BI-04 harbours an ATP-binding cassette (ABC) transport system.² The binding protein *BLAXBP*, which is associated to this ABC transporter binds a range of arabinosyl-decorated and undecorated xylo-oligosaccharides, with preference for tri- and tetrasaccharides. Crystal structures of *BLAXBP* in complex with four different ligands revealed the basis for this recognition as the transport protein is able to bind oligosaccharides in two opposite orientations, which facilitates the optimization of interactions with the various ligands. Binding site plasticity is further enhanced by a spacious binding pocket accommodating decorations at different main chain positions and conformational flexibility of a lid-like loop. Phylogenetic and genetic analyses show that *BLAXBP* is highly conserved within *Bifidobacterium*, but is lacking in other gut microbiota taxa. These novel data highlight the role of glycan uptake in establishing metabolic specialization of bifidobacteria and in contributing to syntrophy (cross-feeding) in arabinoxylan metabolism amongst different taxa in the gut niche.

Acknowledgements

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The modular intramolecular *trans*-sialidase from *Ruminococcus gnavus* ATCC 29149 suggests a novel mechanism of mucosal adaptation in the human gut microbiota

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The gastrointestinal (GI) mucus layer is colonized by a dense community of microbes catabolizing dietary and host (mucin) carbohydrates during their expansion within the gut. Alterations in mucosal carbohydrate availability impact on the composition of microbial species including enteric pathogens.

The species *Ruminococcus gnavus* is a commensal anaerobe present in the GI tract of $\geq 90\%$ of humans and has been implicated in gut-related diseases such as inflammatory bowel diseases (IBD). The ability of *R. gnavus* strains to grow on GI mucin is strain dependent and relies on the expression of a potential GH33 glycoside hydrolase, as shown by comparative genomics and transcriptomics¹.

Here we show using a combination of enzymology and crystallography, that this enzyme is an intramolecular *trans*-sialidase (IT-sialidase) which allows the bacteria to cleave off terminal sialic acid residues from mucins, releasing 2,7-anhydro-Neu5Ac instead of free sialic acid². The IT-sialidase is specific for α 2-3 linked sialic acid conjugates although it can accommodate different moieties at the non-reducing end, as determined by High Performance Anion Exchange Chromatography-Pulsed Amperometric Detection (HPAEC) and Nuclear Magnetic Resonance (NMR). The sialidase consists of a GH33 catalytic domain and a carbohydrate binding domain CBM40. The contribution of both domains to the activity and unusual substrate specificity of the enzyme will be discussed in light of X-ray structures.

Using bioinformatics approaches, we showed evidence of IT-sialidases in human metagenome sequence data with a prevalence in samples from IBD metagenomes². Our results uncover a previously unrecognised enzymatic activity in the gut microbiota, which may contribute to the adaptation of intestinal bacteria to the mucosal environment in health and disease.

Acknowledgements Bernard Henrissat for help with CAZy domain boundaries.

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Galactomannan degradation by *Bifidobacterium*

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The gut microflora is important for the wellbeing of the host. The microflora keeps out pathogenic bacteria, stimulates the immune system and produces nutrients that can be used by the host [1]. It is therefore of interest to study how dietary fibers affects the composition of the gut flora and how gut bacteria metabolize fibers, including enzyme-catalyzed hydrolysis.

We have studied the effect on the caecal bacteria composition and metabolic responses in rats fed with low and high fat diets in combination with guar galactomannan of low, medium and high viscosity. The low viscosity guar galactomannan was prepared by enzymatic hydrolysis of guar galactomannan using a β -mannanase from *Aspergillus niger*. *Bifidobacterium* sp. were upregulated when the rats were fed guar galactomannan regardless of viscosity [2]. Some of the genome sequenced bifidobacteria harbor β -mannanases, the main enzymes responsible for galactomannan degradation. We have cloned and characterized a GH26 β -mannanase, BaMan26A, from *Bifidobacterium adolescentis* ATCC15703.

BaMan26A is active on guar and carob galactomannans and crystalline mannan from ivory nut. The enzyme is modular, containing apart from the catalytic module an immunoglobulin-like and two carbohydrate binding modules of family 23 which bind to carob galactomannan (Kd 8.8 mg/l) [3]. The main products from carob galactomannan were found to be mannotriose and digalactosylated mannotriose. These produced mannoooligosaccharides were used as a carbon source for *in vitro* growth studies of *B. adolescentis*.

The growth studies of *B. adolescentis* showed no uptake of the mannoooligosaccharides, but the digalactosylated mannotriose, one of the dominant products from carob hydrolysis, seemed to be degraded into mannotriose and galactose, which could be utilized by *B. adolescentis* to grow.

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Understanding complex glycan utilization in the human microbiota

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The human large bowel is colonized by a community of microbes, the microbiota, which has a significant impact on human health and nutrition through the production of short chain fatty acids (SCFAs), and by interaction with the host immune system. The major nutrients available to these organisms are dietary glycans, also known as complex carbohydrates. Thus, dietary and nutraceutical strategies, based on complex carbohydrates, can, potentially, be deployed to encourage the dominance of beneficial microbes in the microbiota, particularly those producing health promoting SCFAs such as propionate and butyrate, and bacteria that have an anti-inflammatory impact through its interaction with the human immune system, ensuring that this microbial ecosystem has a positive influence on human health. This approach, however, is greatly restricted by a critical lack of understanding of the mechanisms by which complex glycans are metabolized by the microbiota. Significantly, the wealth of genomic and metagenomic microbiota sequence presents an exciting, but so far unfulfilled, opportunity to make decisive advances in our understanding of glycan metabolism in the human large bowel. This seminar will review our biochemical, genetic and microbiological strategies, in harness with metagenomic and genomic data, to understand the mechanisms of complex glycan utilization by the human microbiota^{1,2,3}. The models established, will trigger the development of novel dietary strategies that are designed to maximize human health through manipulation of microbiota structure.

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Poster Presentations

Anticoagulant activity of sulfated polysaccharide-rich macroalgae extracts

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Macroalgae have been used in traditional medicine for centuries and perceived as a food with great health benefits. Recently, several studies have shown that macroalgae possess a variety of biological activities (Mohamed et al. 2012; Andrade et al. 2013) and are capable of synthesizing a wide range of key nutrients such as proteins, minerals, and fibers (Lahaye 1991; Fleurence 1999). Macroalgae are also an important source of polysaccharides, such as sulfated polysaccharides, which are the source for many of their potential properties that might find relevance in nutraceutical, pharmaceutical and cosmeceutical applications (Jiao et al. 2011). Nowadays, to treat health troubles linked to thrombo-embolic complication, most of the anticoagulant treatments are heparin-based. Despite its major anticoagulant activity, heparin can cause serious adverse events. Moreover, its low bioavailability makes such a treatment really expensive. Given the risks and high costs of these treatments, there is a compelling need for further investigation of new sources of anticoagulants. To study the anticoagulant potential of sulfated polysaccharides from macroalgae, different extracts were prepared using various extraction processes leading to enriched polysaccharides fractions. Extracts from six edible seaweeds, including brown (*Laminaria digitata*, *Fucus vesiculosus*, *Himanthalia elongata*, *Ascophyllum nodosum*), green (*Ulva lactuca*), and red (*Chondrus crispus*) macroalgae, were prepared and the biochemical composition of each extract was determined. The potential anticoagulant activity of each extract was also investigated using different scales, from the specific antithrombin-dependent pathway (anti-Xa and anti-IIa) to the intrinsic and/or common (Activated Partial Thromboplastin Time), extrinsic (Prothrombin Time) or common (Thrombin Time) anticoagulant pathways. Furthermore, the extract anticoagulant properties were compared with those of commercial anticoagulants: heparin and Lovenox®.

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Elucidating the impact of N-glycosylation on the ability of recombinant CBM3 from *Clostridium thermocellum* to modify pulp and paper properties

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The hydrolytic activity of the enzymes traditionally used in paper industry to modify pulp and paper properties often leads to the reduction of fiber strength [1]. Thus, the use of the carbohydrate-binding modules (CBMs) of these enzymes has emerged as an interesting alternative, as CBMs have been shown to enhance the surface/interface properties of cellulose fibers independently from the catalytic domain [2].

The CBM3 from the *Clostridium thermocellum* CipA scaffolding protein was previously reported to improve pulp drainability when conjugated with polyethyleneglycol (PEG), which was used to mimetize glycosylation [3]. Otherwise, it didn't affect any of the pulp and paper properties studied [3]. Although not glycosylated in nature, this CBM3 has three potential N-glycosylation sites. Therefore, to elucidate the impact of N-glycosylation on its ability to modify the properties of cellulose fibers, we assessed the effect of two versions of this CBM3 recombinantly produced in *Pichia pastoris*, one non-glycosylated and other glycosylated, on the properties of *Eucalyptus globulus* pulps and handsheets.

Although glycosylation reduced the CBM3 adsorption (16%) and affinity (35%) to Avicel (microcrystalline cellulose), as well as the CBM3 ability to promote the hydrophobization of Whatman's paper surface, the two CBM3 versions affected the *E. globulus* pulp and paper properties in a similar way. None of the CBM3s altered the drainability of *E. globulus* pulps, but both improved significantly the burst and tensile strength indexes of *E. globulus* handsheets, by up to 12% and 10%, respectively. This yet unknown capacity of CBM3 to improve paper strength-related properties was dependent on the amount of CBM3 used, but not on its glycosylation. Therefore, our results show that the N-glycans attached to CBM3 did not significantly change its effects over pulp and paper properties.

Acknowledgements

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Discovery and characterization of novel carbohydrate esterases

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In the context of a biobased economy, valorizing most of sugars contained in the plant cell walls becomes a crucial target. One step towards achieving this goal will be to advance in pentoses extraction, a neglected aspect of biorefining compared to hexoses utilization. Most of pentoses are contained in hemicelluloses, which represents up to 40% of lignocellulosic biomass and therefore a huge sugar reservoir. Many hemicelluloses are highly substituted by esterified phenolic or acetic acids which contribute to the indigestibility of the polysaccharides (1, 2). Removal of these substitutions by carbohydrate-acting esterases (CEs) such as feruloyl esterases and acetyl esterases exposes glycosidic linkages, playing an important role in the biomass deconstruction to enhance enzymatic cocktails.

While many interesting fungal CEs have already been studied, little is known about bacterial CEs. In previous study a functional analysis of metagenomics libraries from invertebrates was performed, revealing numerous bacterial hemicellulose active-enzymes (3). Regarding CEs, 63 sequences encoding novel putative CEs were revealed, with 20 CEs classified in the CAZy database belonging to the families CE1, CE4 and CE7. These enzymes have been cloned and expressed in *E. coli*, and subsequently characterized in order to evaluate their activity on model and complex substrates. Several multimodular enzymes composed of glycosyl hydrolases, carbohydrate binding modules and esterase domains were identified, cloned and expressed. Here we present the potential properties of the most original and promising enzymes.

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Hydrolysis of xylan by thermophilic family 10 xylanase in the presence of biomass-dissolving ionic liquids

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Xylanases are used in lignocellulose hydrolysis to increase the accessibility of cellulolytic enzymes to cellulose and cleave xylo-oligosaccharides, which can be inhibitors for cellulolytic enzymes. Ionic liquids (ILs) are studied in the pretreatment of lignocellulose hydrolysis. However, the remaining ionic liquids can be detrimental to the enzymes used in the hydrolysis. Therefore, finding cellulases and hemicellulases that remain active in the presence of residual ILs is necessary for efficient processing of lignocellulose with IL pretreatment and enzymatic hydrolysis. We have studied GH11 and GH10 xylanases for their tolerance towards ILs (Li et al., 2013; Chawachart et al., 2014). Thermostable GH10 xylanase from *Thermopolyspora flexuosa* (TfXYN10A) turned out to be one of the most tolerant xylanases. It tolerated partly 15-35% water solutions of the tested ILs (Fig. 1). The presence of ILs reduces the temperature optimum of the enzyme, but at 80°C the half-life was even increased in the presence of 15% and 35% of [EMIM]OAc and 15% [DBNH]OAc (Table 1).

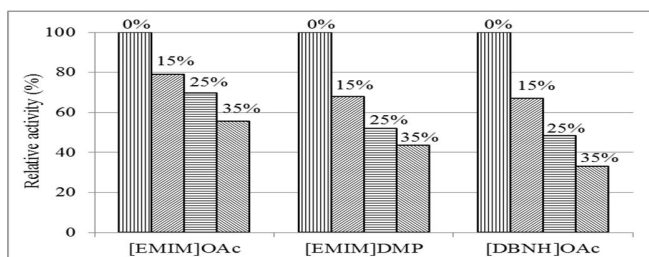


Fig 1. Effect of biomass-dissolving ionic liquids on TfXYN10A. Reaction conditions were 2 h at 60 °C, pH 5, with 1% xylan as substrate.

Table 1. Half-life (min) of Tf XYN10A at 80°C, pH-5.

IL (%)	[EMIM]OAc	[EMIM] DMP	[DBNH]OAc
0 %	64.9 + 2.5	64.9 + 2.5	64.9 + 2.5
15 %	97.8 + 4	50.9 + 3.2	85.6 + 4.3
35 %	94.3 + 2.1	45.7 + 2.8	46.7 + 3.7

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Swollenin from *Trichoderma reesei* exhibits hydrolytic activity against cellulosic substrates with features of both endoglucanases and cellobiohydrolases

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The cellulolytic and hemicellulolytic enzymes of *Trichoderma reesei* comprise one of the best characterised enzyme systems involved in lignocellulose degradation. In this work, swollenin (SWOI), a protein recognised based on its sequence similarity with plant expansins, has been characterised [1]. As most of the *T. reesei* cellulases, SWOI is composed of two modules, an N-terminal cellulose binding module (CBM) connected by a linker region to an expansin-like catalytic module [2]. SWOI and its catalytic domain were subjected to analysis of their hydrolytic activity on different soluble carbohydrate polymers. By measuring the production of reducing ends, zymogram-, and viscosity analysis, SWOI was shown to have activity on substrates containing β -1,4 glucosidic bonds, *i.e.* carboxymethyl cellulose, hydroxyethyl cellulose and β -glucan. The formation of oligosaccharides from β -glucan was analysed by HPLC and showed cellobiose as the main reaction product. SWOI was also able to hydrolyse soluble cello-oligosaccharides and the products formed were all consistent with SWOI cleaving a cellobiose unit off the substrate. In conclusion, the *T. reesei* swollenin showed a unique mode of action with similarities with action of both endoglucanases and cellobiohydrolases.

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Characterization of a GH62 α -L-arabinofuranosidase from *Aspergillus nidulans*: Linking functional diversity with phylogenetics

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L-arabinofuranose (Araf) residues are abundant in plant cell walls as L-arabinan chains of pectin and side chains in arabinoxylan, arabinogalactan and gum arabic. Removal of Araf residues is important in complete degradation of plant material in various industrial processes such as bioethanol production (1). The present *Aspergillus nidulans* L-arabinofuranosidase (AnAbf62A-m2,3) of glycoside hydrolase family 62 (GH62) displays unusually high activity for low viscosity wheat arabinoxylan and moderate ability to hydrolyse sugar beet L-arabinan and 4-nitrophenyl- α -L-arabinofuranoside. NMR studies revealed that AnAbf62A-m2,3 hydrolyses Araf from singly α -1,2 and α -1,3-substituted xylose residues releasing α -1,3-Araf three times faster than α -1,2-Araf. It has been shown that arabinoxylans that contain singly α -1,2 and α -1,3-Araf have to be oriented oppositely for hydrolysis (2). The slower rate of α -1,2-Araf release may thus reflect that arabinoxylan binds weaker in the orientation that is compatible with hydrolysis of α -1,2-Araf from xylose of the backbone. NMR analysis further showed that AnAbf62A-m2,3 releases product with inverted anomeric configuration, as for the GH43 (3), the other clan GH-F member (RW.ERROR - Unable to find reference:33).

Finally, phylogeny and analysis with the Peptide Pattern Recognition (PPR) tool revealed that sequences within the GH62 are divided into three distinctive subfamilies, which differ from the two subfamilies, which the GH62 family was previously divided into (5). The PPR analysis further revealed sequence motifs that distinguish the three subfamilies, which might be important for the functionality of the GH62s within each subfamily.

Acknowledgements

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Efficient chemoenzymatic synthesis of antioxidants using feruloyl esterases in detergentless microemulsions

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Feruloyl esterases (FAEs, E.C. 3.1.1.73) have been proved appealing biocatalysts not only for the degradation of plant biomass but also for the modification of compounds with potential use in food, cosmetic and pharmaceutical industries [1-4]. The enzymatic synthesis of biologically active compounds with antioxidant activity using carbohydrate esterases as biocatalysts allows more flexible process configurations such as lower temperatures (50-60°C) comparing to the counterpart chemical process (150°C), one step production of one product instead of mixtures and no need of by-product and catalyst residues removal.

Aim of this work is the optimization of reaction conditions for the efficient synthesis of the following biologically active compounds: prenyl ferulate, prenyl caffeate, glyceryl ferulate and 5-*O*-(trans-feruloyl)-arabinofuranose. The products were obtained by the enzymatic transesterification of a ferulate ester with aliphatic alcohols or sugars and present improved hydrophobicity/hydrophilicity comparing to the starting antioxidant. For boosting the FAEs' synthetic pathway, non-conventional media with low water content are required. For this purpose, a ternary system of n-hexane/alcohol/water forming detergentless microemulsions [5] was selected, where reaction conditions were optimized regarding the best temperature, solvent composition and substrate/enzyme ratio. The possibility of implementing alternative reaction media such as ionic liquids, organic solvents, oil-in water microemulsions was also evaluated.

Acknowledgements

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Alkyl mannosides produced by alcoholysis with β -mannanases from the fungi *Trichoderma reesei* and *Aspergillus nidulans*

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Biomass has the potential to substitute fossil resources, the main cause of anthropogenic climate change, in numerous sectors, e.g. energy and chemicals. It is, therefore, important to proceed the development of biotechnology. Alkyl glycosides are a group of biodegradable and non-toxic compounds with surfactant potential. It is possible to enzymatically synthesise alkyl glycosides from carbohydrates and alcohols with glycoside hydrolases [1]. With the aim to produce a set of alkyl mannosides possessing different properties, three fungal β -mannanases were selected based on the difference in hydrolysis products and previously shown alcoholysis capacities; *TrMan5A* and its mutant R171K from *Trichoderma reesei* and *AnMan5C* from *Aspergillus nidulans* [2,3]. Alkyl mannosides produced by the enzymes were detected with matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF-MS). With methanol as acceptor and mannotetraose as donor substrate *AnMan5C* produced methyl mannobioside as the unique alcoholysis product and showed the highest efficiency of alcoholysis. With *TrMan5A* R171K it was possible to synthesise methyl mannotrioside to a significant extent. Ongoing work using hexanol as acceptor have resulted in the synthesis of hexyl mannobioside by both *TrMan5A* and *AnMan5C*. Forthcoming work intends to use an organic solvent to increase the yield of hexyl mannosides. A decrease in water activity can favour alcoholysis over hydrolysis as seen in a study of a β -glucanase where acetonitrile was used [4]. Moreover, hexyl mannobiose was detected with reversed phase chromatography. This technique is planned to be further used together with nuclear magnetic resonance (NMR) to quantify this new potential surfactant that is environmentally friendly produced.

Acknowledgements

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Development of microbial production processes for levan polysaccharide

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Levan is a $\beta(2-6)$ -linked fructose homopolysaccharide that is extracellularly produced from sucrose-based substrates by a variety of microorganisms. As a microbial EPS, levan has valuable properties like water solubility, strong adhesivity, film-forming ability, low viscosity, high solubility in oil, compatibility with salts and surfactants, stability to heat, stability to acid and alkali, high holding capacity for water and chemicals, and good biocompatibility(1). Due to its outstanding properties levan that is mainly associated with high-value applications, has a high commercialization potential for various sectors like pharmaceutical, chemical and food industries and there is a growing body of interest in research associated with levan and its applications. Since it is microbially produced using mesophiles at very high production costs, economic hurdles need to be overcome (2). Hence development of novel microbial production processes for levan holds great industrial importance. Recently, halophilic bacterium *Halomonas smyrnensis* AAD6^T has been reported as a high-level levan producer extremophile for the first time by our research group. Considering the advantages associated with the use of a halophilic producer organism and high industrial potential of levan biopolymer, research activities were focused on developing feasible and low-cost production processes. Using both conventional and systems based approaches production yields in semi-chemical media were substantially improved. Improved production yields with concomitant reduction in production costs were obtained using various biomass resources and sucrose containing sugary wastes as low-cost substrates. Moreover, genome-scale metabolic model of *Chromohalobacter salexigens* was refined for reconstruction of the generic metabolic model of *H. smyrnensis* AAD6^T to elucidate the relationship between levan biosynthesis and other metabolic processes. Results of the metabolic systems analysis indicated the stimulatory effect of mannitol on levan biosynthesis which was further verified experimentally (1). Furthermore, the systematical analysis was performed to figure out the effects of basal medium components and boric acid was found to be the most effective stimulator of levan production (2).

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The financial support of The Scientific and Technological Research Council of Turkey (TUBITAK) through project 114M239 is gratefully acknowledged.

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Roles of starch and sucrose in exopolysaccharide formation by *Lactobacillus reuteri*

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Oral diseases such as dental caries and periodontitis occur when the equilibrium of the indigenous bacteria is compromised in the oral cavity (1). A strong correlation has been established between the saliva lactobacilli count and salivary constituents with dietary sugars (2). Glucansucrase (GS) enzymes secreted from lactic acid bacteria play an important role by converting sucrose into α -glucan exopolysaccharides (EPS) that function in dental biofilms (3, 4). Maltose and malto-oligosaccharides, produced from starch by salivary α -amylase, also act as acceptor substrates during α -glucan synthesis by GS. The combination of sucrose and starch is potentially more cariogenic than either compound alone. Glycoside hydrolase family 70 includes not only GS enzymes, but also the recently described 4,6- α -glucanotransferase (4,6- α -GTase) enzymes which are inactive with sucrose but cleave (α 1-4) linkages in starch (hydrolysates) and synthesize α -glucans with increased percentages of (α 1-6) linkages (5). Both GS and 4,6- α -GTase enzymes exclusively occur in lactic acid bacteria. Occasionally, they are found in the same strain, e.g. GS GTFA and 4,6- α -GTase GTFB in *Lactobacillus reuteri* 121 (5).

Besides the previously characterized GS GTFA EPS35-5 produced from sucrose, feeding *L. reuteri* 121 with starch hydrolysates as carbon source also resulted in EPS synthesis produced by 4,6- α -GTase GTFB. SEC results showed that the size of both EPS products reached up to 50 MDa. *In vitro* incubations of the *L. reuteri* 121 GTFA plus GTFB enzymes with sucrose plus starch (hydrolysate) yielded initial oligosaccharides and EPS with different structures compared to the products obtained from either substrate alone, evident from 1D/2D ¹H and ¹³C NMR, TOFMS, as well as HPAEC analyses. To sum up, EPS formed by *L. reuteri* 121 varies, reflecting the individual GS GTFA (with sucrose) and 4,6- α -GTase GTFB (with starch hydrolysates) activities, but also the cooperation of both enzymes in the presence of both major dietary sugars, sucrose and starch.

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Towards the set-up of a recombinant protein production facility for fungal carbohydrate-active enzymes using the yeast *Pichia pastoris*

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Filamentous fungi are the predominant source of lignocellulolytic enzymes used in industry for the transformation of plant biomass into high-value molecules and biofuels. The rapidity with which new fungal genomic and post-genomic data are being produced is vastly outpacing functional studies. This underscores the critical need for producing enzymes lacking confident functional annotation, a prerequisite to their functional and structural study. The yeast *Pichia pastoris* has become increasingly popular as a host for the production of fungal carbohydrate-active enzymes (CAZymes). Therefore, the objective of this study was to try to further streamline recombinant protein expression in *Pichia pastoris*.

Fungal CAZymes glycoside hydrolase (GH) 5, GH11, and GH45 (Couturier et al., 2011 a and b) were used to evaluate the following steps of the protocol set-up by InvitrogenTM for expressing recombinant proteins in *P. pastoris*: (i) expression constructs, (ii) selection and (iii) culture of transformants, (iv) protein purification and (v) expression conditions. The main achievements reported in this study are (i) a plating-free selection of transformants using frozen cells, (ii) the miniaturization of liquid cultures to deep well microplate format using both inducible (AOX1) and constitutive (GAP) promoters and (iii) the automated purification of secreted His-tagged recombinant proteins.

We have set-up a shorten protocol for expressing fungal CAZymes in *P. pastoris* that is applicable to other proteins.

Acknowledgements

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Structural analysis of chitin oligosaccharide deacetylases – the “subsite capping model”

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Chitin deacetylases (CDAs) catalyze the hydrolysis of the acetamido group in GlcNAc residues of chitin, chitosan, and chito oligosaccharides (COSs). A major challenge is to understand how CDAs specifically define the distribution of GlcNAc and GlcNH₂ moieties in the oligomeric chain. The particular acetylation patterns of natural COSs dictate their role in cell signaling and other biological activities.

We present here the structural bioinformatics analysis of this family of enzymes that lead us to the proposal of a model to rationalize the de-*N*-acetylation specificity exhibited by different CDAs: the “subsite capping model” [1]. According to this model, the acetylation pattern exhibited by different CDAs is governed by critical loops that shape and differentially block accessible subsites in the binding cleft of these enzymes.

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The abstract has been withdrawn

HEXPIN: Hetero-exopolysaccharide – milk protein interactions

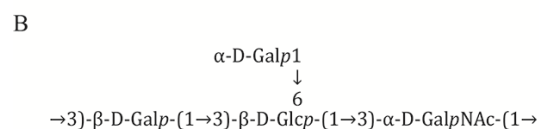
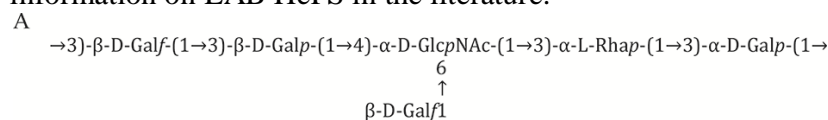
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Today's food industry often uses restricted chemically modified polysaccharides (PS) in dairy products as thickeners and stabilizers. Hetero-exopolysaccharides (HePS) excreted by various lactic acid bacteria (LAB) strains are *generally recognized as safe* (GRAS) and have a similar positive impact on the textural properties in fermented milk products [1]. The textural effect stems in part from associative complexation between HePS and milk proteins, both caseins and whey proteins which complex with the casein micelle in milk during thermal processing. However, the molecular basis for the HePS-protein interactions and impact on textural properties is poorly understood and there is demand for understanding the HePS-protein interaction in order to be able to naturally increase viscosity in the final consumer product.

We produced and purified a series of HePS of known structures in yields of 41–135 mg l⁻¹ from different *Streptococcus thermophilus* strains and 8–10 mg l⁻¹ from *Lactobacillus rhamnosus* GG grown in skimmed milk medium. By using surface plasmon resonance (SPR), binding of the HePS to the whey proteins β -lactoglobulin or α -lactalbumin was monitored at different pH, ionic strength and temperature. HePS from *Lactobacillus rhamnosus* GG (A) and *Streptococcus thermophilus* Sfi6 (B) demonstrated an optimum in the binding capacity around pH 4.0 and 70 mM NaCl and decreasing binding capacity with increasing temperature in the range 15–35°C. Interaction at pH 4.0 between HePS A and B and native or heat denatured β -lactoglobulin was supported by retardation in affinity gel electrophoresis. As a tool for exploring the potential of the structural variations represented by HePS, we established a database of structural and biophysical properties based on information on LAB HePS in the literature.



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A single point mutation near the active center is responsible for high efficiency of the *Thermotoga maritima* α -galactosynthase in the synthesis of known amylase substrate

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Alpha-amylase is an enzyme that helps to digest starch and glycogen. It is produced mainly by exocrine pancreas and salivary glands. For many years, the determination of the level of alpha-amylase in serum and plasma has been used in patients to diagnose acute pancreatitis. Early methods were based on the titration of iodine and a change in the optical density of the highest dilution of a complex between iodine and starch, cleavable by alpha-amylase. Now, most of the methods are based on the production of *p*-nitrophenol from a saturated oligosaccharide substrate with blocking groups attached to the ends of sugar molecules. The maltooligosaccharides with chromophoric group are perspective substrates for the α -amylases. They don't need the additional enzymes for their hydrolysis, so that further polymerization of products and substrate doesn't occur. Here, we present enzymatic approach for the synthesis of known substrate for the α -amylase based on the use of the modified α -galactosidase from the hyperthermophilic bacteria *Thermotoga maritima*. This enzyme is well studied and the α -galactosynthase derived from it was recently reported [1]. Recently, we have reported that GH family 36 α -galactosidase from *Thermotoga maritima* demonstrates ability to form different α -galactosidic linkages and predicted several single amino acid substitutions leading to changes in the transglycosylation patterns of this enzyme [2,3]. On the basis of simple assumptions made during molecular modeling, residues were identified whose mutation could yield increased (1,2)-regioselectivity of transglycosylation. Two mutations indeed produced mainly α (1,2)-linked galactosides using *p*-nitrophenyl α -D-galactopyranoside as a substrate in self-condensation reactions. Here, we present a simple protocol for the synthesis of 2-chloro-nitrophenyl α -galactomaltoside using α (1,2)-specific galactosylsynthase produced on the basis of the enzyme and show a role of the P402 in the binding of the acceptor, 2-chloro-4-nitrophenyl-maltoside, in the enzyme active center.

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Insights into LPMO diversity from structural and functional characterization of NcLPMO9C, a broad-specificity lytic polysaccharide monoxygenase

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Recently it was shown that oxidative hydrolysis of cellulose is catalyzed by so called Lytic Polysaccharide MonoOxygenases (LPMOs)[1] which play an essential role in plant biomass degradation along with well-studied cellulases[2-4]. LPMOs are copper-dependent biocatalysts that use molecular oxygen and an electron donor to break glycosidic bonds[5-7]. Electrons may be provided by a reducing agent such as ascorbic acid, gallate or reduced glutathione, or by a co-secreted enzyme such as cellobiose dehydrogenase. We have conducted structural and functional studies of an LPMO from *Neurospora crassa*, NcLPMO9C, that is unique in that is active on soluble cello-oligosaccharides and hemicellulosic substrates, in addition to cellulose[8]. NcLPMO9C is a two-domain protein, containing an N-terminal LPMO-domain and a family 1 carbohydrate-binding module (CBM1) that are connected through a serine- and threonine-rich linker comprising approximately 50 residues. The crystal structure of the catalytic domain shows an extended and highly polar substrate-binding surface that conceivably has the possibility to interact with a variety of sugar substrates. The structure also revealed features that are likely responsible for the strict oxidative regioselectivity of NcLPMO9C (C4 oxidizing). The unique ability of NcLPMO9C to act on soluble substrates was exploited to study enzyme-substrate interactions. ITC studies revealed binding affinities in the low micromolar range for polymeric substrates. Truncation of the CBM1 reduced these affinities and the activity on polymeric tamarind xyloglucan, while activity on phosphoric acid-swollen cellulose did not change. Analyses of the copper site by EPR spectroscopy, copper affinity determinations by ITC, and determination of the redox potential yielded results similar to those obtained previously for cellulose-active LPMOs, indicating that the unique functionality of NcLPMO9C is primarily due to its extended polar substrate-binding surface and, to some extent, the CBM1.

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How to quantify enzyme activity and kinetics in "non-bulk" systems? An example through the enzymatic hydrolysis of hemicellulose thin films

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In the carbohydrate-based bioindustry, enzymes are often used in conditions where they have to work on solid surfaces and/or penetrate within structures that are locally highly concentrated. The effect of such physical constraints on the enzyme activity and kinetics is however poorly understood; mostly because following and quantifying the hydrolysis in such conditions is still a challenge.

With this work, our intention is to provide a detailed characterization of an enzyme's activity when its substrate is both concentrated and immobilized at a solid interface. This is essentially done by monitoring the *in-situ* degradation of a thin film of a model hemicellulose using a Quartz Crystal Microbalance with Dissipation (QCM-D).

The thin film is composed of a unique arabinoxylan, extracted from wheat bran, and that is chemically modified for covalently binding onto gold.¹ The film is partly swollen by water, and its water content (hence its local dry concentration) can be tuned by partially removing the L-arabinofuranosyl units that decorate the xylan chain.² The film is then put into contact with a solution containing an endo-1,4- β -xylanase (*NpXyn11A*³), into the QCM-D cell, and the loss of mass in the film is followed with time as degradation occurs. Using mathematical models that are under development in our laboratory, we aim at converting the raw QCM-D data into kinetics curves that give the reaction rate as a function of the polymer concentration in the film. Such a procedure would allow us to accurately compare the behavior of the enzyme in a film with its "bulk" behavior; the latter having been characterized classically with a dilute solution of the same substrate. Our results should reveal precious indications about the effect of substrate immobilization and conformation/concentration on the action of an enzyme.

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The CBMomes of cellulolytic bacteria colonizing different ecological niches present distinct carbohydrate specificities

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Plant cell walls, predominantly composed of cellulose, are the most abundant source of organic carbon on earth. The energetic constraints posed by anaerobic ecosystems lead to the evolution of a remarkable highly efficient supramolecular enzyme complex, termed cellulosome¹. Cellulosomes are multi-enzyme complexes of Carbohydrate Active enZymes (CAZymes) and play a pivotal role in the degradation of plant cell wall carbohydrates. *Clostridium thermocellum* and *Ruminococcus flavefaciens* are two highly efficient cellulolytic bacteria that produce very complex but well characterized cellulosomes. The two bacteria colonize different, highly dynamic and populated ecological niches, the soil and the rumen of mammals, respectively. Here we have cloned, expressed and purified all known Carbohydrate Binding Modules (CBMs), the CBMome, of *C. thermocellum* and *R. flavefaciens*. Ligand specificity of the two CBMomes was analysed using a carbohydrate microarray platform^{2,3} derived from target polysaccharide glycomes. This technique enabled the screening of a wide range of protein-carbohydrate interactions in a high-throughput manner using only minute amounts of sample. The data revealed that the two bacteria present CBMomes expressing different carbohydrate-binding capacities. Overall this work suggests that the physico-chemical properties of different ecological niches modulate the evolution of CAZymes presenting distinct ligand specificities.

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Determination of mammalian sialic acids in infant formula

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Dietary sialic acids are important for critical immune system and cognitive development in infants. Although these functionalized neuraminic acids are present in all mammalian milk, the proportions and amounts are species specific. Human milk contains sialyl-conjugates with N-acetylneuraminic acid (Neu5Ac) but not N-glycolylneuraminic acid (Neu5Gc). In contrast, bovine milk has primarily Neu5Ac, but also a small proportion of oligosaccharides possessing Neu5Gc. Additionally, bovine milk provides 75% less total sialic acid content than that of human milk. As th, infant formula manufacturers have begun enriching infant formula products with sialic acids. Sialic acids determinations in a complex matrix such as dairy products can be challenging. In addition sialic acids are conjugated and must be released for determinations. Adding another level of complexity, these conjugates are different: oligosaccharides in human milk and glycoproteins for infant formula. Acid hydrolysis is typically used to release the bound sialic acids. However additional carbohydrates are also released causing analytical interferences which can lead to inaccurate reporting of sialic acid concentrations. Sialic acid and other carbohydrates are poor chromophores and therefore require costly, and labor intensive derivitization for spectrophotometric detection. High performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) is a well established direct sensitive and selective method to determine carbohydrates. Here we compare and contrast using ion-exchange and enzyme digestion as sample preparation methods. Sialic acids are determined by HPAE-PAD.

Cellobiohydrolase and endoglucanase respond differently to surfactants during the hydrolysis of cellulose

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Non-ionic surfactants such as polyethylene glycol (PEG) can increase the glucose yield from enzymatic saccharification of pretreated lignocellulosic substrates. In a hydrolysis study using Avicel and PASC, we have shown that the boosting effect of PEG differs for the individual cellulases. For both substrates during hydrolysis with a monocomponent exo-cellulase CBH1, the presence of PEG led to an increase in the final glucose concentration, while there was no change in hydrolysis yield with an endoglucanase (EG). Also, PEG had no effect on the activity of β -glucosidases. PEG increased the thermal stability of both the CBH1 and the EG, but only the activity of the CBH1 increased in the presence of PEG. We propose that this has to do with either the processive mechanism of CBH1 affecting the k_{cat} , or a change in the adsorption/desorption rate (k_{off}) of the CBH1.

We have also observed that the properties of the hydrolysis liquid phase change when PEG is present in solution. Using LF-NMR relaxometry we found that PEG increases the relaxation time of the hydrolysis liquid phase, i.e. water is more active. Thus, CBH1 seems to benefit from water being more active, while this is not the case for EG. Based on these results, the properties of the liquid phase have a direct impact on the hydrolysis yield using commercial cellulase mixtures, as CBH1 makes up most of the *T. reesei* secretome from which most cellulolytic enzyme mixtures are produced industrially.

From waste to health care product: Pectic oligosaccharides produced from citrus peels by treatment of endo-pectate lyase (PL1B) inhibiting colon cancer cells

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An endo pectate lyase (PL1B) from *Clostridium thermocellum* ATCC 27405 cellulosome was cloned and expressed as a 40 kDa protein. It was found to be active on pectic substrates and mainly produced unsaturated di- and tri-galacturonates after enzymatic cleavage of polygalacturonic acid and pectin [1]. The natural pectin was isolated from waste citrus peels of *Citrus limetta* (Sweet lemon) by acid extraction, yielding 12% powdered pectin. The structure of isolated natural pectin was characterized by NMR spectroscopy and was found to contain 77% methylation in the polysaccharide chain. Enzymatic treatment of the isolated natural pectin by PL1B produced unsaturated di-galacturonate as the major product. This was confirmed by Thin Layer Chromatography (TLC) analysis after running the enzymatic reaction samples of different time intervals from 0 to 60 min. The oligosaccharides were then separated by gel filtration using Bio-Gel P2 matrix, where deionized water was used as the mobile phase. The purified oligosaccharides were free from any other contaminants and were freeze dried and used for further studies. The effect of these oligosaccharides at varying concentrations from 0.05 to 0.5 mg/ml was tested on colon cancer cell lines (HT-29) for 3, 6, 12, 24 h. The population of viable cells present after the treatment was measured by MTT assay, where the cell viability is measured by the colorimetric changes. The results showed the 70% inhibition of the growth of HT-29 cells by 0.5 mg/ml oligosaccharides after treatment for 24 h. This result was further verified by observing the changes in the cell morphology upon the treatment of oligosaccharides. Light microscopic images of 0.5 mg/ml oligosaccharides treated HT-29 cells showed disintegrated cell membrane as compared to the untreated cells. Pectin being large polymeric compound is difficult to get absorbed by the alimentary canal hence enzymatic degradation facilitates the production of smaller oligosaccharides which gets easily absorbed by the alimentary canal. Hence, the pectic oligosaccharides can be produced on a large scale by the recombinant endo pectate lyase degradation of citrus peels. The citrus peel which is generally considered as waste can serve as source for a potent healthcare commodity.

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Enzymatic synthesis of lipid II and analogues

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The emergence of antibiotic resistance has prompted active research in the development of antibiotics with new modes of action. Among all essential bacterial proteins, transglycosylase polymerizes lipid II into peptidoglycan and is one of the most favorable targets because of its vital role in peptidoglycan synthesis. Described in this study is a practical enzymatic method for the synthesis of lipid II, coupled with cofactor regeneration, to give the product in a 50-70% yield. This development depends on two key steps: the overexpression of *MraY* for the synthesis of lipid I and the use of undecaprenol kinase for the preparation of polyprenol phosphates. This method was further applied to the synthesis of lipid II analogues. It was found that *MraY* and undecaprenol kinase can accept a wide range of lipids containing various lengths and configurations. The activity of lipid II analogues for bacterial transglycolase was also evaluated.

Modification of cell wall glucuronoxylans by expressing a GH115 α -glucuronidase in *Arabidopsis thaliana*

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Expressing microbial polysaccharide-modifying enzymes *in planta* is an attractive approach to tailor lignocellulose properties and to study the importance of specific cell wall structure to plant. Glycoside hydrolase family 115 α -glucuronidase (AGU) from white rot fungus, *Schizophyllum commune* cleaves the internal α -D-(4-O-methyl)glucopyranosyluronic acid ((Me)GlcA) from O-acetylglucuronoxylans (AcGXs) or xylooligosaccharides¹. Therefore, the enzyme can be an interesting tool to modify the structures of AcGXs present within the cell walls. In this work, the *ScAGU115* was constitutively expressed in model plant, *Arabidopsis thaliana*. The recombinant *ScAGU115* was active on the internally substituted aldopentaouronic acids. Surprisingly, the cell wall (Me)GlcA and other non-cellulosic sugars, as well as the lignin content, remained unchanged. In contrast, disrupting the endogenous glucuronyltransferases was shown to be effective reducing the (Me)GlcA substituents in *gux1gux2* mutant plants². The stem section of *ScAGU115*-expressing plants exhibited a decreased signal from UX1 antibody that recognizes (Me)GlcA substituents, the signal was not affected by the transgene when the sections were deacetylated prior to labeling. In contrast, *gux1gux2* mutant lacked the UX1 signals in both native and deacetylated cell walls. This indicates that the *ScAGU115* affects only those (Me)GlcA moieties, which are accessible to UX1 antibody, and constitute a small fraction in AcGXs of *Arabidopsis*. However, majority of the (Me)GlcA substituents were resistant to *ScAGU115* modification, likely due to the shielding of neighboring acetyl substitutions. Therefore, a better strategy for tailoring side groups of AcGXs *in planta* can be co-expressing with an acetyl xylan esterase to harness synergism between these side groups removing enzymes.

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Biochemical characterization of a new GH-70 enzyme from *Leuconostoc citreum* NRRL B-1299

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Glucansucrases (GS) from glycoside hydrolase family 70 (GH70) are transglucosylases found in several lactic acid bacteria from the genera *Leuconostoc*, *Lactobacillus*, *Streptococcus* and *Weissella*. They catalyze the polymerization of glucosyl residues from sucrose, an economical and abundant agrosresource. Depending on the enzyme specificity, the synthesized α -glucans vary in terms of size, structure, types of glucosidic bonds and degree of branching. This composition variety confers to α -glucans diverse properties, making them useful for several applications in food and pharmaceutical industries¹.

The sequencing of *Leuconostoc citreum* NRRL B-1299 genome allowed the identification of a new GS called DSR-M, which is highly similar (94% sequence identity) to the recently identified GSE16-5 glucansucrase from *L. citreum* LBAE-E16². This 229kDa enzyme has the particularity to synthesize a low molar mass dextran (around 27kDa) composed of exclusively α -(1 \rightarrow 6) linkages.

To allow the structural and biochemical characterization of this enzyme, a truncated version was constructed, expressed and purified. Kinetic study and crystallization trials are in progress. The original results recently obtained will be presented and discussed.

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Discovery of novel carbohydrate active enzymes for plant biomass degradation by metagenomics of hyperthermophilic communities

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Hyperthermophilic microorganisms, most of them belonging to the domain of Archaea, thrive at temperatures >80°C and attract remarkable interest in basic and applied research for the uncommon intrinsic stability of their enzymes. In the framework of the discovery of new enzymes for biocatalysis and biotransformation for industrial applications, we investigate, through a metagenomic approach, the complex communities of hyperthermophiles populating volcanic sites to understand their adaptation and evolution to extreme conditions and to exploit them as a rich source of novel biocatalysts [1, 2].

A metagenomic analysis of the microbial communities in two neighboring mud/water pools in the solfataric field of Pisciarelli, Agnano (Naples, Italy), showed that the *phylum* Crenarchaeota was prevalent, but differentially abundant at the genus level in the two pools that differ in T and pH (pool1 T=85°C and pH 5.5; pool2 T=94°C and pH 1.5, respectively). 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. Total DNA was extracted from the microbial community growing in the enriched samples and, again by metagenomics, we determine the full set of carbohydrate active enzymes (CAZymes) of these microbial communities. The detailed phylogenetic and physiological analysis of the enriched samples and their CAZymes family composition will be discussed.

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Structural and functional investigation of a lytic polysaccharide monooxygenase (LPMO) by NMR spectroscopy

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Lytic polysaccharide monooxygenases (LPMOs) are abundantly present in biomass-degrading microbes and are currently classified as auxiliary activity auxiliary activity (AA) families 9 (AA9, formerly GH61), 10 (AA10, formerly CBM33) and 11 (AA11) (see www.cazy.org⁴). LPMOs have been shown to catalyze the cleavage of glycosidic bonds in chitin and cellulose through hydroxylation of either carbon within the scissile bond and are thus likely to play important roles in future biorefining^{3, 5-7}. However, the molecular basis of their unprecedented catalytic activity remains largely unknown. We have used NMR techniques to address structural and functional aspects of BILPMO10A, a chitin-active AA10. NMR structural and relaxation studies showed that the core of BILPMO10A is a compact β -sandwich with a flat surface that includes the protein N-terminus. These observations are in agreement with previously investigations on other AA10 LPMOs^{1, 2, 8}.

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A novel carbohydrate esterase isolated from an Arctic environmental metagenome

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Carbohydrate-active enzymes (CAZymes) are involved in the metabolism and synthesis of saccharides. Degradative CAZymes form part of the hemicellulolytic and cellulolytic enzyme systems of microbes which break down plant biomass, and have possible applications in biotechnology (1).

Here we describe the biochemical characterisation of a family 15 carbohydrate esterase (CE-15) 'MZ0003' which was cloned from a metagenomic DNA library derived from marine arctic sediment. MZ0003 appears to be of bacterial origin, and has low sequence homology (20%) with previously characterized GH15 enzymes from mesophilic and thermophilic fungi.

In silico and in vitro studies indicate that MZ0003 has structural and biochemical properties that differ from these fungal homologs. In particular, MZ0003 has a broader acetylated substrate range and lower temperature optimum than the previously characterized CE-15 enzymes. A study of the inhibition of activity by site-directed mutagenesis was used to investigate the role of predicted active site residues in catalysis, while a combination of homology-based modelling and preliminary crystallization studies are being undertaken to provide more structural information.

These studies on MZ0003 represent the first instance of biochemical characterization of a bacterial member of the GH15 CAZy family.

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Towards monoglycosylation of organic molecules with glucansucrases: reaction –and enzyme engineering

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Glucansucrases (GS) are exclusively found in lactic acid bacteria and catalyze the conversion of sucrose into α -glucan polysaccharides. However, in the presence of suitable acceptor substrates, GS also use sucrose as a donor substrate to glycosylate these acceptors. Glycosylation can significantly improve the physicochemical and biological properties of small molecules like vitamins, antibiotics, flavours and fragrances. Typically, the solubility and stability of the glycosylated compounds are increased, improving also their bioavailability¹.

Recently, we observed that various GS glycosylate a wide range of phenolic and alcoholic compounds. Moreover, it was shown that GS are capable of adding multiple glucosyl moieties to one acceptor molecule, producing glycosides of different size and structure. On the one hand multiglycosylation leads to an extra increase in solubility and stability of the glycosylated organic molecules. On the other hand, however, multiglycosides lose most of their functional activity. Consequently, monoglycosylation is generally preferred over multiglycosylation.

Two strategies were followed in order to push the reaction towards monoglycosylation: reaction – and enzyme engineering. The former strategy consisted of selecting the optimal reaction conditions. It was found that high acceptor concentrations lead to relatively more monoglycosylation. However, the obtained results were not satisfying yet and therefore the biocatalyst itself was optimized by means of mutagenesis of L981² and W1065², residues involved in the positioning of saccharide acceptors in the active site. The resulting mutants lost most of their ability to accept saccharides while retaining a high specificity for phenolic and alcoholic molecules. Hence, the conversion of monoglycosides into multiglycosides was blocked. Furthermore, by eliminating α -glucan synthesis, the most important side reaction, higher glycosylation yields were obtained. Altogether, this led to an improvement in monoglycosylation yield of 50% for several phenolic and alcoholic compounds (catechol, resorcinol, hydroquinone, butanol, hexanol and octanol), indicating the general applicability of this strategy.

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The feruloyl esterase gene family of *Aspergillus niger*

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Ferulic acid (FA) is a major phenolic acid component of plant cell walls. FA and to a lesser extent other hydroxycinnamic acids (e.g., *p*-coumaric acid) are covalently linked mainly to xylan and pectin through ester linkages. They cross-link the cell wall polysaccharides to themselves and to the phenolic polymer lignin. This increases the physical strength and integrity of plant cell walls as well as reduces their biodegradability by microorganisms. Feruloyl esterases (or ferulic acid esterases, FAEs) [E.C. 3.1.1.73] are responsible for the release of FA from plant cell walls. As they are able to liberate phenolic acids from natural plant sources as well as agro-industrial by-products, FAEs are widely used in the food, feed, pulp-paper, and pharmaceutical industries as well as in biofuel production. These broad application fields require various types of FAEs to fit specific pH, temperature, and other conditions.

Aspergillus niger is one of the most well-known and important industrial fungi, which produces a wide range of plant cell wall-degrading enzymes. Among others, two FAEs from *A. niger* (AnFaeA and AnFaeB) have been characterized and are among the most thoroughly studied FAEs. Phylogenetic analysis of fungal FAEs performed by Benoit et al. (2008) has led us to numerous novel FAE candidates including several from *A. niger*. In this study, the putative FAEs from *A. niger* were investigated using phylogeny, gene expression and enzyme characterization. Highlights from this study will be presented.

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Structural and functional studies of a *Fusarium oxysporum* cutinase with polyethylene terephthalate modification potential

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Cutinases (EC 3.1.74, categorized in CE family 5 of the CAZy database), are enzymes that degrade plant polyester waxes, like suberin and cutin, the major constituent of plant cuticle. The cuticle shields leaves, shoots and other exposed parts of plants from microorganisms such as fungi and bacteria. Cutinases are employed by plant pathogens to deconstruct the protective cuticle and then invade the plant by secreting an armory of carbohydrate degrading enzymes (1). Recent findings have revealed that cutinases are capable of hydrolysing or modifying a variety of synthetic esters and polyesters such as polyethylene terephthalate (PET), a property which renders them interesting biotechnological targets. Moreover, degradation products, some of which are found almost exclusively in the suberized plant cell walls, could have considerable value as sources of oleochemicals. Here we report the expression, characterization and crystal structure of *FoCut5a*, a cutinase from the mesophilic fungus *Fusarium oxysporum*. The recombinant enzyme was heterologously expressed either in the cytoplasmic or periplasmic space of *Escherichia coli*, taking advantage of the *pelB* signal sequence. The crystal structure of *FoCut5A* was determined at 1.9 Å resolution and solved by molecular replacement, using *Fusarium solani* cutinase structure (PDB code 1CEX) as starting model (2). The two enzymes have high sequence identity and their overall structures are very similar, however, there are small differences between the structures that can potentially explain the variations in the biochemical properties of the two enzymes. *FoCut5A* has a maximum activity at 40 °C, when tested on the three *p*-nitrophenyl synthetic esters of aliphatic acids (C₂, C₄, C₁₂), with the highest catalytic efficiency found against the butyl ester. *FoCut5A* was also found capable of hydrolyzing PET model substrates, indicating a potential use of this enzyme in surface modification of synthetic polymers.

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The hydrophilic character of cytotoxic payloads affects functional properties of antibody-drug conjugates

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Antibody-drug conjugates (ADCs) were prepared using closely related auristatin and dolastatin payloads that have different hydrophilic character. The formed ADCs and the free drugs were characterized in biochemical and cellular assays. Free drugs with increased hydrophilic character were less cytotoxic to cancer cells when applied to the cell culture medium of cancer cells, reflecting their diminished ability to pass through the cell membrane compared to more hydrophobic drugs. However, when conjugated to antibodies (as ADCs), the payloads triggered similar cytotoxicity as their hydrophobic counterparts. Therefore, by increasing the hydrophilicity of the ADC payloads, ADCs with less cytotoxicity to non-target cells can be constructed. Hydrophilic drugs are also known to have less bystander efficacy, *i.e.* ability to attack nearby cells after destroying a target cell. We propose that by selection of a suitable hydrophilic ADC payload, both safety to non-target cells and bystander effect can be optimized.

With these prerequisites, ADCs with high drug-to-antibody ratios (DAR > 4) were generated. With these ADCs, it was demonstrated that the more hydrophilic payloads improved the ADCs stability (less aggregation) and biocompatibility, while the cytotoxic activity remained unaltered. In conclusion, the choice of payload chemistry can affect functional properties in addition to the direct cytotoxicity of ADCs.

Assisting effect of a carbohydrate binding module on Glycosynthase-catalyzed polymerization

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The development of polysaccharides have been of great interest for their use in biomaterials and biomedical applications. As alternative to source extraction and chemical synthesis, enzymatic synthesis is a powerful synthetic strategy. Particularly, the glycosynthase technology has provided a broad range of oligosaccharides, glycoconjugates and polysaccharides [1]. Artificial polysaccharides with regular and defined structure and, in some cases crystalline morphology, have been obtained using glycosynthase-catalyzed polymerization of simple activated glycosyl donors [2,3]. The donor is self-condensed by the non-nucleophilic glycosynthase mutant and polymer products can be elongated until they become insoluble.

We envisioned the *in vitro* application of non-catalytic carbohydrate binding modules (CBMs) in the glycosynthase polymerization reaction. In nature, CBMs are found as discrete domains of polysaccharide degrading enzymes where they play critical roles in the recognition of inaccessible polysaccharides and potentiate the activity of their cognate catalytic modules against insoluble substrates [4]. These considerations prompted us to explore the glycosynthase-catalyzed polysaccharide synthesis in the presence of CBMs. CBMs could enhance the solubility of the growing polymeric chain formed during the reaction, increasing the turn-over of the enzyme and obtaining insoluble polymers with probably higher molecular weights and lower polydispersion indexes. We combined a glycosynthase derived from *B. licheniformis* 1,3-1,4- β -glucanase [2] and the CBM11 domain of *Clostridium thermocellum* Lic26A-Cel5E enzyme, which binds β -1,3-1,4-glucans [5]. We here report the effects of the CBM11 domain as a free protein or fused to the catalytic glycosynthase domain on the formation of these mixed-linked glucans.

We acknowledge Dr. Harry Gilbert, Newcastle University, for providing the CBM11 gene. VC acknowledges a predoctoral fellowship from IQS. This work was supported by grant GLYCOZYMES (BIO2013-49022) from MICINN, Spain.

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Crystallographic studies of a member of the lytic polysaccharide monoxygenase family AA13

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Lytic polysaccharide monoxygenases (LPMOs) are a new class of copper dependent carbohydrate modifying redox enzymes that have gained much interest in the last ten years [1, 2, 3, 4]. Four families of LPMOs are currently classified in CAZY under Auxiliary Activities [5]. AA9 (previously GH61), AA10 (CBM33) and AA11 [6] have all so far been found to act on polysaccharides containing β -1,4-linkages. Recently however, a family of proteins previously shown in the patent literature to boost amylolytic activity [7], has been confirmed to be an LPMO family (AA13) [8, 9] degrading starch and related polysaccharides, which are α -1,4-linked. Many of the enzymes belonging to AA13 have an associated starch-binding CBM (Carbohydrate Binding Module).

In this contribution we focus on the insight in substrate specificity provided by crystallographic studies on a member of the family naturally devoid of CBM, AoAA13 from *Aspergillus oryzae* [9].

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Endogenous degradation activity for slimy extracellular polysaccharide produced by *Lactobacillus fermentum* TDS030603

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L. fermentum TDS030603 secretes highly viscous extracellular polysaccharides (EPS) into culture media [1]. In our laboratory, an eventual decrease of viscosity of the culture medium has been observed during the stationary and death phases without any fragmentation of the EPS main chain. Recently, Gerwig et al. defined chemical structures of the EPS, and its branched side chains turned out to be heterogeneous [2]. Based on these facts, we hypothesized that side chains of the EPS should be degraded during cultivation that evokes decrease of the culture media viscosity, hence this study was conducted to explore EPS degradation activities of *L. fermentum* TDS030603.

To investigate approximate localization of the EPS degradation activity, crude protein fraction was prepared from the culture supernatant by 80%-saturation ammonium sulphate precipitation, and cell-free extract and cell debris were prepared as follows: cells of *L. fermentum* TDS030603 were harvested after 48 h of static incubation at 30°C, washed with phosphate-buffered saline (PBS) three times, sonicated for 1 min with 30 sec intervals up to 5 min on ice, and centrifuged at 7000 g for 30 min at 4°C. To extract cell-associated substances from *L. fermentum* TDS030603, harvested cells were treated with 2 M guanidine hydrochloride at 37°C for 2 h, or with 0.2 M glycine, 1 M lithium chloride, 40 µg/ml lysozyme, and PBS each at 4°C for 1 h. EPS degradation reaction was carried out at 30°C, for appropriate periods, at pH 4.5, using 0.009% (w/v) of thoroughly purified EPS as substrate. Subsequently, the reaction mixtures were frozen immediately at -80°C and kept until used. After removal of remained EPS in the reaction mixture by ultrafiltration using YM-3 device, 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatives of mono- and oligo-saccharides were prepared according to the literature [3] and analyzed on high-performance liquid chromatography (HPLC).

Single peak corresponding to standard galactose was observed on HPLC chromatogram only when the cell debris was mixed with the EPS substrate. This peak appeared when the reaction was performed at pH 4.5, but not at pH 7.0. All the five extracts from the cell-surface gave several peaks of PMP-derivatives including the one corresponding to the standard galactose. Among the five, highest peaks were observed toward the extract with 0.2 M glycine, and areas of some of the peaks increased as the reaction time prolonged. The EPS degradation activity of the glycine extracts was abolished by heat treatment at 100°C for 10 min. These results indicated presence of EPS-degrading substance(s) on cell-surface of *L. fermentum* TDS030603.

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Activity studies on lytic polysaccharide monooxygenases

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Lytic polysaccharide monooxygenases (LPMO, former GH61) are enzymes disrupting crystalline cellulose by cleaving glycosidic bonds¹. However, kinetic studies are difficult due to the lack of fast screening methods. The presented activity assay² applies the ability of LPMO to produce hydrogen peroxide in absence of substrate. The produced hydrogen peroxide can be followed spectroscopically using horseradish peroxidase and Amplex red. Presence of substrate decreases production of hydrogen peroxide, limiting substrate studies, but allowing a large array of screenings targeting activity enhancers, inhibitors and optimal conditions. Additionally, a substrate assay³ based on oxidation of phosphoric acid swollen cellulose by LPMO, followed by high performance anion exchange chromatography was used. Combining both assays, LPMO was studied regarding potential substitutes for molecular oxygen and reducing agents. Furthermore, molecular dynamics simulations were performed to study the interaction of the enzyme with its cellulose substrate showing multiple interaction sites (Fig. 1).



Fig. 1: Molecular dynamics simulation with LPMO (PDB ID 2YET) and cellulose, overlay of all frames. LPMO is shown as cartoon with residues located in the active site or assumed to interact with the substrate drawn as sticks. The cellulose strands are indicated by strings with hexagons.

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Characterization of a broad substrate specificity AA9 lytic polysaccharide monoxygenases from *Podospora anserina*

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Plant cell walls, made up of cellulose and hemicelluloses, represent a high potential renewable resource for the production of 2nd generation biofuels and platform molecules for the chemical industry. In the last few years, significant progress in enzymatic polysaccharide degradation was achieved with the identification of a new class of fungal secreted enzymes, the lytic polysaccharide monoxygenases (LPMOs) that facilitate the degradation of cellulosic biomass. AA9 LPMOs (formerly classified into the GH61 family) are copper-dependent enzymes found exclusively in fungi. They act on the relatively crystalline surface regions of their substrates (mainly cellulose) by oxidizing at different positions of the glycosidic bond in cellulose. Here, we characterized in depth a family AA9 from *Podospora anserina* (*PaLPMO9H*) with biochemical and biophysical approaches. This copper monoxygenase, which bear a family 1 Carbohydrate Binding Module (targetting cellulose) at its C-terminus was expressed heterologously using the methylotrophic yeast *Pichia pastoris* and produced in bioreactor. Using chromatographic and fluorescent methods, we showed that *PaLPMO9H* is versatile in terms of substrate specificity as it displayed activity on cellulose and β -(1,4)-linked hemicellulose polysaccharides (xyloglucan and mixed-linkage β -glucan). Ability to oxidatively cleave cello-oligosaccharides was also demonstrated. Sequence similarities and differences with the *Neurospora crassa* NcLPMO9C (Isaksen et al., 2013; Agger et al., 2014) will be presented. The broad specificity of *PaLPMO9H* makes it attractive to tackle the recalcitrance of lignocellulosic biomass.

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Molecular cloning, expression and characterization of novel endo- β -1, 4-mannanase of a family 10 glycoside hydrolase from *Pedobacter saltans* DSM12145

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Microbial mannanases have become biotechnologically important since they target the hydrolysis of complex polysaccharides of plant tissues into simple molecules like manno-oligosaccharides and mannose. The role of mannanases in the paper and pulp industry is well established and more recently they have been reported being applied in the food and feed industry. β -Mannanase is an excellent biocatalyst for the production of gluco-manno-oligosaccharides (GMOS). Several studies have confirmed that GMOSs with the degree of polymerization (DP) of 2 to 6 have the health benefits on humans. GMOS could increase the growth of intestinal microorganisms, decrease the populace of pathogenic bacteria and improve the integrity of intestinal mucosa [1]. This study reports an endo- β -1,4-Mannanase (*PsMan*) from *Pedobacter Saltans* DSM 12145 belonging to family 10 glycoside hydrolase. The nucleotide sequence encoding *PsMan* was accessed from the CAZy (Carbohydrate Active Enzymes, www.cazy.org) database [2]. *PsMan* was amplified by PCR, the amplicon was cloned into pET-28a(+) vector and expressed in *E. coli* BL21 (DE3) cells. The recombinant endo-1,4- β -mannanase was soluble protein and showed a homogeneous protein of molecular size, approximately, 43 kDa band after purification by immobilized Ni^{2+} ion affinity chromatography. The specific activity of the purified *PsMan* was 15.08 U mg^{-1} using Konjac-glucomannan as substrate. *PsMan* displayed optimal temperature, 37°C and optimum pH, 6.5. The K_m and V_{max} values for Konjac-glucomannan, are 0.255 mg mL^{-1} and 15.67 $\mu\text{mol min}^{-1} \text{mg}^{-1}$, respectively. The presence of 1 mM of Cu^{2+} ions increased the activity of *PsMan* by 2 fold. Analysis of hydrolyzed products showed that *PsMan* could hydrolyze different types of mannan based polysaccharides, releasing manno-oligosaccharides. The main products released were mannobiose, mannotriose and other higher oligosaccharides which confirmed that *PsMan* is an endo-acting enzyme.

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Insights into the mechanism of glucuronoxylan hydrolysis revealed by the 3-dimensional crystal structures of glucuronoxylan-xylanohydrolase (*CtXyn30A*) from *Clostridium thermocellum*

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The family 30 glycoside hydrolase (GH30) from *Clostridium thermocellum* is a modular carbohydrate active enzyme. The full length enzyme *CtXynGH30* contains an N-terminal catalytic module, *CtXyn30A* followed by a family 6 carbohydrate binding module, *CtCBM6* and a dockerin type I at the C-terminal [1]. *CtXyn30A* was cloned, expressed and purified as a soluble ~45 kDa protein. *CtXyn30A* preferably hydrolysed xylan based substrates like beechwood-, birchwood- and glucurono-xylan and displayed optimum activity at pH 6.0 and at 70°C. Zymogram analysis of *CtXyn30A* with beechwood xylan confirmed its xylanase activity. *CtXyn30A* gave maximum activity of 30.9 U mg⁻¹ with beechwood xylan. Kinetic studies of *CtXyn30A* with beechwood xylan gave a K_m of 0.2184 mg mL⁻¹ and K_{cat} of 1.205×10⁵ min⁻¹. The TLC analysis of hydrolysed products of beechwood xylan by treatment with *CtXyn30A* produced a series of higher xylo-oligosaccharides showing that it is an endo-xylanase. Whereas, on prolonged treatment of xylohexaose by *CtXyn30A* did not lead to formation of any lower xylo-oligosaccharides or monomer xylose. This suggested that *CtXyn30A* does not act on linear xylo-oligosaccharides and has an absolute requirement of substitution of glucuronic acid or 4-O-methyl glucuronic acid on xylans for enzymatic cleavage. Crystallization of *CtXyn30A* was achieved by sitting-drop vapour diffusion method and X-ray structure was solved by molecular replacement using the crystal structure of XynC from *Bacillus subtilis* 168 (PDB code 3gtn) as search model [2]. The three dimensional structure of *CtXyn30A* display a (β/α)₈ TIM barrel core with a side associated β-sheet domain. Structural data collected from crystals grown in different conditions showed that *CtXyn30A* can bind a variety of ligands such as the proteolysed His-tag peptide, HEPES, glycerol, tartaric or malonic acid in the catalytic site. The analysis of the residues interacting with the various ligands bound in the *CtXyn30A* structures suggested that Trp81, Tyr139, Trp143, Gln173, Tyr200, Tyr227, Ser231, Trp264, Tyr265, Arg268 and Tyr270 are important for ligand binding. A comparison with homologous structures solved in complex with different ligands confirmed that the equivalent residues are involved defining a consensus catalytic site. Thus, *CtXyn30A* catalytic site displays specificity pockets either side of active site, enabling GH30-8 members to bind to a number of different ligands that may mimic the different decorations of the xylan backbone.

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Enhanced saccharification and effective pretreatment of corn cob by utilizing recombinant cellulase and hemicellulase from *Clostridium thermocellum* for bioethanol production

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Exploitation of readily available agro waste such as corn cob (*Zea mays* var. *saccharata*) is one of the most promising renewable energy sources for production of bioethanol. Cost-effective saccharification and fermentation of lignocellulosic biomass encompasses the efficiencies of i) pretreatment, ii) carbohydrate degrading enzymes and iii) fermentative microbes. The present study involves the comparative analysis of two individual pretreatments viz. wet oxidation and ammonia fiber expansion (AFEX) and a mixed pretreatment strategy involving both, for efficient hydrolysis by recombinant cellulase and hemicellulase from *Clostridium thermocellum*. The analysis of structural carbohydrates content of untreated corn cob revealed cellulose, 52.2 (% w/w); hemicellulose, 28.0 (% w/w) and lignin 14.3 (% w/w), whereas after the mixed pretreatment (wet oxidation and AFEX) the content was 49.3, 23.0 and 6.2 (% w/w), respectively, displaying significant removal of lignin content. Fourier transform infrared (FT-IR) spectroscopic and field emission scanning electron microscopy (FESEM) analyses confirmed the structural deterioration with increased porosity and lignin breakdown of mixed pretreated corn cob. *E. coli* cells containing recombinant family 5 glycoside hydrolase (GH5) a cellulase [1] or GH43 a hemicellulase from *Clostridium thermocellum* [2] were used in saccharification process for the release of hexose and pentose sugars from pretreated lignocellulosic biomass. 1% (w/v) Simultaneous saccharification and fermentation (SSF) trials with individual pretreatment strategy of wet oxidation and AFEX comprising enzymatic consortium of GH5 and GH43 along with fermentative microbes, *Candida shehatae* and *Saccharomyces cerevisiae* gave 1.4 g/L and 1.2 g/L ethanol titres, respectively. However, improved ethanol titre of 1.7 g/L and yield of 0.235 (g of ethanol/g of substrate) was obtained with mixed pretreated 1% (w/v) biomass. Increasing the substrate concentration to 5% (w/v), SSF shake flask contributed a 5.5-fold higher ethanol titre (9.30 g/L) and yield (0.257 g of ethanol/g of pretreated substrate).

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Structural and functional studies of a copper-dependent lytic polysaccharide monooxygenase from *Bacillus Amyloliquefaciens*

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The enzymatic degradation of polysaccharides is a major goal of the biotechnology industry, most notably for both first (starch) and second (cellulose/chitin) generation biofuel processes. In recent years, the production of second generation biofuels has become increasingly more important to prevent unnecessary food usage. In addition, more efficient techniques of degradation of these second generation materials have become a requirement for scientific research. One such focus for increased efficiency is the class of copper-containing enzymes discovered in 2010¹, known as Lytic Polysaccharide Monooxygenases (LPMOs). These enzymes were originally thought to possess no catalytic activity, so were incorrectly classified at the time of discovery. They were re-classified as “Auxiliary Activity” enzymes in 2013² (AA9 and AA10). The aim of my work is to study the bacterial LPMOs from the AA10 family, to determine their substrate specificity and ultimately oxidative mechanism. The enzyme I am currently working on is from the bacterium known as *Bacillus amyloliquefaciens*, the 3-D structure of which had been solved previously.³ Here I showcase the enzymatic degradation of chitin by the *BaAA10*, as well as structural and mechanistic studies using various techniques, including EPR and X-ray crystallography.

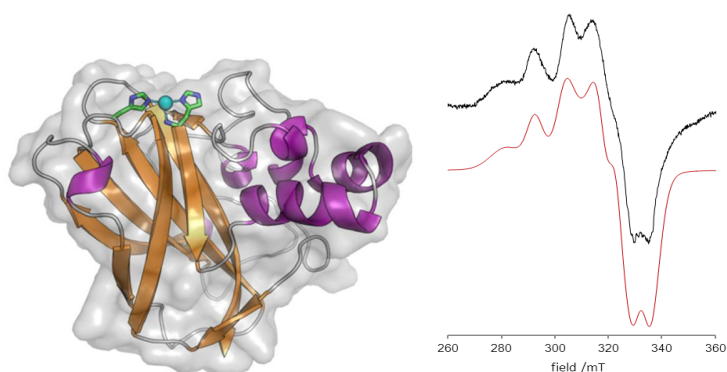


Figure 1. 3-D structure and EPR spectrum (black – raw data; red – simulated) of *BaAA10*.³

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This work is funded by the BBSRC White Rose Doctoral Training Partnership.

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Metagenome mining of novel enzymes for the bioethanol industry

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Lignocellulosic biomass is considered an attractive and immediate source for liquid biofuel. The hydrolysis of lignocellulose requires the synergistic actions of glycoside hydrolases (GHs), thus, novel enzymes with enhanced activity and production are necessary to improve the economics of the process. In the framework of this research, metagenomics and biochemical approaches were combined to identify and characterize novel enzymatic systems geared for lignocellulose degradation.

Our view on the GHs world is restricted to the current known sequences available in the databases. Thus, when analyzing metagenomes with conventional methods novel GHs genes with low sequence similarities are usually not identified.

Our sequence-based screening was designed to elicit novel GH genes from metagenomic data. We designed our search algorithm under three principles: a) a GHs designated algorithm, rather than a full data analysis; b) Genomic Neighborhood approach assimilation for sieving excess data ; and c) Conserved Neighborhood approach for putative novel GH genes evaluation .

Our search algorithm managed to reduce the analyzed data, and allowed us to focus on several clusters, which potentially harbor novel GHs. Following the scan of 75 thermal-springs metagenomes, 97 DNA scaffolds with 778 hemicellulolytic genes have been identified. Two clusters with putative cellulose degradation pathways were chosen for further evaluation and three putative novel GHs had taken for biochemical analysis. To-date, we identified a gene product with CMCase activity in each cluster, belonging to families GH5 and GH44. These results supports our working hypothesis that these clusters are involved in cellulose hydrolysis. Several of the remaining genes in the clusters are potentially new GH families and are in the process of biochemical characterization.

This work was supported by the Grand Technion Energy Program (GTEP), and comprises part of The Leona M. and Harry B. Helmsley Charitable Trust reports on Alternative Energy series of the Technion, Israel Institute of Technology, and the Weizmann Institute of Science.

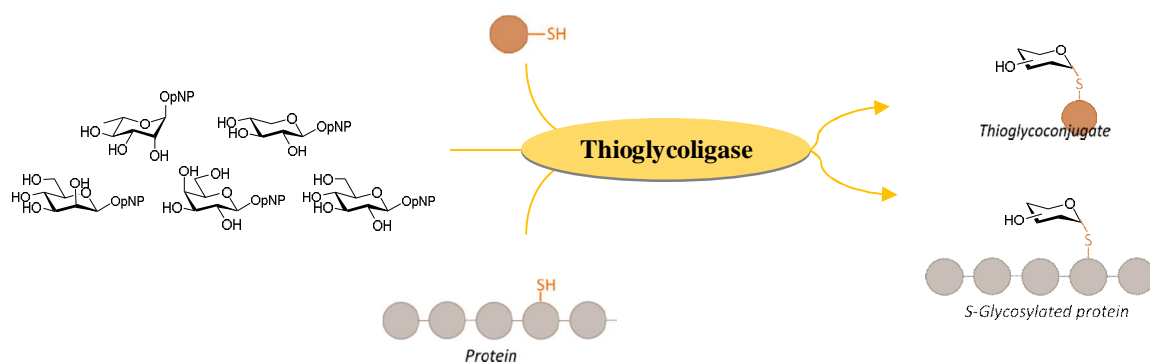
Thioglycoligases : innovative biocatalytic tools for S-glycosylated proteins synthesis

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Glycosylation is one of the most complex co- and post-translational modifications of proteins and is involved in numerous biological processes as protein folding, metabolic stability, intracellular trafficking... As a consequence, errors in glycosylation have dramatic impacts on those processes affording apparition of several diseases (diabete, ancer, auto-immune disease...).¹ During the last past decades, advances made on carbohydrate chemistry allowed synthesis of glycoproteins and new glycoconjugates in order to understand their role and generate new therapeutics. Amongst them, thioglycoconjugates, in which a sulfur atom has replaced the glycosidic oxygen atom, became of great importance through their ability to resist to chemical and enzymatic cleavage.² The ambition of our project is therefore to develop a novel method based on chemoenzymatic synthesis to access S-glycosylated proteins.



Recent advances on protein bioengineering highlighted a new class of biocatalysts, thioglycoligases³, able to generate thioglycosidic linkage. In order to take up this challenge a bank of thermophilic mutated GHs from *D. thermophilum* was developed. All enzymes were assayed for thioglycosylation using a range of various thiols as glycoside acceptors. At the end, best thioglycoligase candidates will be used to generate S-linked glycosylated amino acid paving the way for S-glycoproteins synthesis.

Acknowledgements This research is supported by Region Centre and Glycodiag funding.

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Rational design of a novel cyclodextrin glucanotransferase from *Carboxydocella* to improve alkyl glycoside synthesis

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Alkyl glycosides have gained huge attention from different industries due to their biodegradability, high surface activity and low toxicity. In many applications defined alkyl glycosides with an elongated carbohydrate moiety is required. The elongation process of an alkyl glycoside can be accomplished by using different cyclodextrins as donors in coupling reactions catalyzed by CGTase [1]. CGTase or cyclodextrin glucanotransferase (EC 2.4.1.19) belongs to GH family 13 and is also capable of catalyzing cyclization, hydrolysis and disproportionation by using substrates like starch. Recently we have characterized a novel CGTase from *Carboxydocella*, which was used to elongate dodecyl- β -maltoside [2]. The high disproportionation reactivity limits the usability of this enzyme for producing well-defined alkyl glycosides (Table 1). In order to improve the synthesis of defined alkyl glycosides we have designed several mutations by a site-directed mutagenesis approach. The objective of this mutagenesis is to increase the coupling product formation while decreasing the disproportionation activity of the enzyme. Currently, we are analyzing the effect of these mutations and comparing them with the wild-type.

Table 1: Coupling and disproportionation activities of CGTase from *Carboxydocella*. Dodecyl- β -maltoside was used as acceptor for coupling and disproportionation.

Enzyme	Donor	Coupling ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)	Disproportionation ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)
CGTase (<i>Carboxydocella</i>)	α -CD	40.5 \pm 0.5	66.0 \pm 10.7
	β -CD	25.2 \pm 1.9	19.6 \pm 4.6
	γ -CD	65.5 \pm 0.7	85.1 \pm 11.2

Aacknowledgements

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Development and application of a synthetic cellulosome-based screening platform for enhanced enzyme discovery

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At the forefront of enzyme discovery, Novozymes continues to search for better ways to screen the growing diversity of glycosyl hydrolases in order to improve enzyme cocktails for biomass conversion. To this end, we have successfully integrated a synthetic biology approach into our next generation of enzyme discovery platforms. Specifically, we have leveraged the available natural diversity of cellulosomal components to develop an artificial mini-cellulosome as the basis for a versatile cell-free method to screen combinations of multiple enzyme activities. In the process, we have engineered a highly thermostable scaffoldin comprised of distinct cohesin domains that stably bind cognate dockerins with high specificity for extended periods under lignocellulose hydrolysis conditions. These protein-protein interactions serve as respective binding modules for covalently-linked libraries of different enzyme classes. Our synthetic cellulosome approach has been adapted to an automated HTS system through which enzymes of synergistic quality are more efficiently identified compared to past conventional approaches. Finally, we have successfully employed this novel screening platform to discover promising new enzyme candidates tailored for improved hydrolytic performance on (AFEX-PCS) an industrially-relevant biomass substrate.

Identification of the catalytic residues of glycosidases from *Paenibacillus thiaminolyticus* as a key into engineering new glycosynthases

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Glycosyl hydrolases β -D-galactosidase and α -L-fucosidase cloned from *Paenibacillus thiaminolyticus*, are interesting not only because of their hydrolase activity but mainly, for their transglycosylation activity, which is important for synthesis of interesting glycosylated molecules. In this project both enzymes were studied using the combination of theoretical and experimental methods. In the theoretical part, the structures of enzymes were predicted and further studies of these structures proposed the possible catalytic residues in both enzymes. The aim of the experimental part is to confirm the predictions of catalytic amino acid residues assumed in the theoretical part. Here we report the final results in which mutated enzymes β -D-galactosidase_mut233, α -L-fucosidase_mut186 and α -L-fucosidase_mut239 were produced and purified. Furthermore β -D-galactosidase_mut157 was purified in earlier experiments. All mutants were tested on several substrates. The results of these activity tests are helpful for better understanding the catalytic machinery of studied enzymes and lead us to possibility of transforming them into glycosynthases i.e. modified glycosyl hydrolases that lack their natural hydrolytic activity, because of mutation of nucleophilic amino acid residue, but maintain their transglycosylation activity. On this poster we also show the first progress in designing of these glycosynthases.

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Identification and characterization of a novel unclassified de-N-acetylase from *Sulfolobus solfataricus*

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In the last few years, our research topic has been focused extensively on the carbohydrate active enzymes from hyperthermophilic Bacteria and Archaea (thermozymes) in order to discover new enzymatic activities, understand the molecular basis of thermal adaptation and, eventually, to design new stabilized biocatalysts for industrial applications [1,2]. Therefore, we embarked on the identification of novel enzymatic activities from the thermoacidophilic Archaeon *Sulfolobus solfataricus*.

Here we report on the identification and the characterization of a hypothetical protein codified by the ORF sso2901. According to BLAST alignments, sso2901 encodes for a putative N-acetylglucosaminyl-phosphatidylinositol-(GlcNAc-PI)-de-N-acetylase (EC 3.5.1.89), which belongs to PIG-L family (Pfam 02585). This family includes the de-N-acetylases that catalyses the second step of GPI anchor biosynthesis [3]. Although the activities on GlcNAc-PI are included in the CAZy database in CE14, sso2901 is not classified in CE families. The biochemical characterization on the recombinant SSO2901 (rSSO2901) on GlcNAc revealed that the maximum activity is observed at pH 8.5 and 70°C, with k_{CAT} 1.94 ± 0.19 s⁻¹, K_M 22.01 ± 4.91 mM and k_{CAT}/K_M 88×10^{-3} s⁻¹ mM⁻¹. In addition, rSSO2901 has shown activity on pNp- α -GlcNAc and pNp- β -GlcNAc, but not on chitobiose, GalNAc, ManNAc, GlcNAc-1P, GlcNAc-6S, UDP-GlcNAc and hyaluronic acid. These results indicate that SSO2901 represents the first de-N-acetylase identified from *S. solfataricus* belonging to PIG-L family. In addition, close to sso2901, we have identified the ORF sso2900, codifying for a hypothetical GT4. Surprisingly, we have observed that the recombinant SSO2900 is able to hydrolyze UDP-GlcNAc. So, we speculate that both enzymes might be involved in the same metabolic pathway and their biochemical characterizations might shed light on their function *in vivo*.

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Development of novel enzymatic tools for the production of xylose-based products within a lignocellulosic biorefinery concept

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Advanced biorefining is a key element of the targeted transition towards a sustainable bioeconomy, which will use plant biomass as a primary carbon source instead of petroleum resources. Utilization of plant biomass is followed by the breakdown of its complex structure into fermentable sugars via a treatment step that uses enzymatic cocktails. Biomass degrading enzymes, such as Glycoside Hydrolases (GHs) [1], often work in synergy with other enzymes and there are numerous examples of natural bi- or multifunctional enzyme assemblies [2]. Microorganisms can be considered as the source of biomass degrading enzymes and the functional screening of their metagenomic libraries, can mediate the discovery of new enzymatic activities [3].

In this project, a novel GH was discovered after the functional screening of a metagenomic library [3] and was annotated as a xylanase. Its amino acid sequence was predicted to also encode a Carbohydrate Binding Module (CBM) [1] and it was then used to construct a 3D structural model. The molecular modeling contributed in understanding the architecture of the domains, revealing that it is a multimodular enzyme consisted of a catalytic GH domain and two CBMs. This novel multimodular enzyme will serve as a blueprint for further enzyme engineering; where the CBM domains could be replaced by another GH, in order to design tailored multifunctional enzymes with multiple catalytic activities for use in biorefineries.

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Biochemical characterization of a novel aldose-ketose isomerase, mannose isomerase from *Marinomonas mediterranea*

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N-acetylglucosamine 2-epimerase (AGE) superfamily enzymes including AGE, aldose-ketose isomerase, and cellobiose 2-epimerase share a catalytic domain formed by an (α/α)₆-barrel and an active-site structure composed by two catalytic His residues acting as general acid and base catalysts. A possible member of AGE superfamily, Marme_2490 from *Marinomonas mediterranea*, is an uncharacterized protein with low amino acid sequence similarity to any characterized enzyme, and its biological functions are unclear. In this study, we investigated biochemical properties of recombinant Marme_2490 produced in *Escherichia coli*.

The recombinant Marme_2490 was successfully produced as soluble protein in *E. coli*, and purified to homogeneity by Ni-chelating column chromatography. From 1 L of the culture broth, 18.8 mg of purified recombinant protein was obtained. To examine the activity for Marme_2490, the protein was incubated with 21 kinds of sugars and the reaction mixture was analyzed on thin layer chromatography. D-Mannose and D-fructose were converted to D-fructose and D-mannose, respectively, indicating that Marme_2490 has D-mannose isomerase activity. Thus, Marme_2490 was designated as *MmMI*. The optimum pH and temperature of *MmMI*, investigated using D-mannose as a substrate, were pH 7.3 and 30° C, respectively. The enzyme was stable in a range of pH 5.5-9.4 and below 35°C after the pH (4° C for 24 h) and heat (pH 7 for 30 min) treatments, respectively. The k_{cat} , K_m , and k_{cat}/K_m of *MmMI* for the epimerization of D-mannose were $463 \pm 3 \text{ s}^{-1}$, $16.7 \pm 1.8 \text{ mM}$, and $27.7 \text{ s}^{-1}\text{mM}^{-1}$, respectively. *MmMI* also had a weak isomerization activity to disaccharides, β 1-4 mannobiose, 4-*O*- β -D-glucosyl-D-mannose, and 4-*O*- β -D-galactosyl-D-mannose (epilactose). Isomerization activity to oligosaccharides has been first found in D-mannose isomerases. In contrast to *E. coli* and *Salmonella enterica* aldose-ketose isomerases (YihS proteins), which catalyze interconversion of D-mannose, D-glucose, and D-fructose [1], *MmMI* did not act on D-glucose (reaction velocity was estimated to be $\leq 0.22 \mu\text{mol}/\text{min}/\text{mg}$ protein, $\leq 0.052\%$ of that to D-mannose) and produce D-glucose from D-fructose and D-mannose. *N*-acetyl-D-glucosamine and cellobiose did not serve as substrates of *MmMI*. Consequently, *MmMI* has been classified to be a member of aldose-ketose isomerase, and it is a novel type of mannose isomerase.

Acknowledgements

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Neopullulanase subfamily and related specificities of the family GH13 - *in silico* study focused on domain evolution

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The α -amylase family GH13 [1] containing ~30 different enzyme specificities and more than 21,000 sequences, represents one of the largest CAZy glycoside hydrolase (GH) families [2]. Earlier, based on a characteristic sequence motif in their 5th conserved sequence region, the two closely related subfamilies, the so-called oligo-1,6-glucosidase and neopullulanase subfamilies were described [3]. Currently the two subfamilies cover several CAZy-defined GH13 subfamilies [4] because the α -amylase family GH13 has been officially divided into 40 subfamilies [5]. The present *in silico* analysis was undertaken in an effort to shed some light on the evolution of modular arrangement within the neopullulanase subfamily (the subfamily GH13_20). The main goal was to reveal the evolutionary relationships between the catalytic domain representing the enzyme specificity and the non-catalytic starch-binding domains (SBDs). The subfamily GH13_20 contains, in addition to neopullulanase, also cyclomaltodextrinase and maltogenic amylase [2-4]. They usually possess the N-terminal SBD classified as the carbohydrate-binding module (CBM) family CBM34 [2,6]. The studied set, extracted mostly from the CAZy database subfamily GH13_20 and family CBM34, consisted of 70 family GH13 members with the emphasis given on biochemically characterized enzymes. The set was further completed by some closely related amylolytic enzymes either from the subfamily GH13_39 having also CBM48 or from GH13_20 with CBM20, or even lacking any CBM.

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Characterization of a GH30 glucuronoxylan specific xylanase from *Streptomyces turgidiscabies* C56

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The gene belonging to glycoside hydrolase family 30 (StXyn30A) was cloned from *Streptomyces turgidiscabies* C56 and the recombinant protein which is a putative xylanase was purified and characterized. This enzyme showed hydrolytic activity on xylans containing glucuronic acid side chains, but not on linear xylooligosaccharides, suggesting that this enzyme positively recognize the glucuronic acid side chain to hydrolyze the main chain of xylan. By thin-layer chromatography and mass spectrometry analysis of GH30 xylanase hydrolysate treated β -xylosidase, it was confirmed that the hydrolysis product from glucuronoxylan has glucuronic acid at the second xylopyranosyl residue from the reducing end.

To understand the mechanism how StXyn30A recognizes glucuronic acid substitution, we focused R296 residue of GH30 from *S. turgidiscabies* C56 which is conserved in bacterial GH30s but not in fungal GH30s. Because molecular modeling analysis and previous studies (ref) suggested the R296 residue in StXyn30A interact with carboxyl group of glucuronic acid side chain of xylan, we constructed R296 mutant enzymes to analyze the activities for glucuronoxylans and hydrolysis products. Significant reduction of the hydrolytic activities for glucuronoxylans were observed in all of R296 mutants. β -xylosidase treatment of hydrolysis products by R296 mutants suggested R296 mutants lose the ability to recognize the glucuronic acid side chains.

Chitinases in a lignin-producing cell culture of Norway spruce

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A suspension culture of Norway spruce (*Picea abies* (L.) Karst.) secretes extracellular lignin into the culture medium (1). Interestingly, a high chitinolytic activity was detected in the medium. Polyacrylamide gel electrophoresis in a chitin-containing gel indicated chitinolytic activity in enzymes of 16–24 kDa, and isoelectric focusing showed the presence of 8–10 acidic and basic chitinolytic enzymes. Some activity was also observed in the higher molecular weight region. Since chitin is not present in plants, we hypothesize that the observed chitinases in the aseptic Norway spruce cell culture may act upon not just chitin but also against endogenous plant substrates.

Chitinases of the culture medium were purified with affinity, size exclusion and ion exchange chromatography. They were characterized by western blotting with antibodies against chitinases. At least group I, II and IV chitinases of 18–35 kDa were observed. The enzyme activity of two cationic chitinases was tested against several cell wall-related polymers using chromogenic polysaccharide hydrogel (CPH) substrates and carbohydrate microarray-based technology. The chromogenic substrates did not reveal any obvious substrate alternatives for the chitinases, but microarray analyses indicated a low mannan response. Currently some of the chitinases expressed in spruce wood and in the cell culture are being cloned and will be produced heterologously. Produced proteins will be further characterized and utilized in substrate analyses in order to eliminate possible contaminant enzyme activities carried over in the purification process of chitinolytic enzymes.

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Enzyme properties affecting enzyme adsorption onto lignin in high solid environments

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Lignocellulosic biomass is a renewable raw material that can be refined to various products including paper, packaging, composites, fuels and chemicals. In the industry, enzymatic hydrolysis of lignocellulosic biomass into fermentable sugars is preferentially carried out in high solid consistency, i.e. over 15 % (w/w) solids concentration¹. Currently there is a limited amount of data available on enzyme performance in high solids concentrations. In high consistency environments enzyme inhibitions are typically stronger, for instance unproductive binding onto lignin. This study aims to elucidate the effect of enzyme properties on unproductive binding in low and high consistency enzymatic hydrolysis. Enzyme binding onto lignin rich residues isolated from steam explosion pretreated spruce and wheat straw was studied with purified *Trichoderma reesei* monocomponent cellulases and a xylanase as well as *Aspergillus niger* β -glucosidase -enzymes. Enzyme binding was studied using conventional adsorption assays as well as quartz crystal microgravimetry technique (QCM). Enzymes containing large hydrophobic patches have been hypothesized to bind more readily onto lignin model films². Nevertheless, thermostable enzymes are expected to be less prone to the presence of lignin³. This paper discusses the effects of enzyme properties, such as surface characteristics and thermostability, on enzyme adsorption and inactivation onto lignin surfaces.

Acknowledgements

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Solution structures of glycosaminoglycans and their complexes with complement Factor H: implications for disease

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Factor H (FH) is a major regulator of complement and is genetically associated with the development of age-related macular degeneration (AMD) in the elderly. Heparin and heparan sulphate (HS) bind to sites found on at least the SCR-6/8 and SCR-19/20 regions of FH. Despite its importance, no molecular structures of free HS and large heparin fragments have been reported. By combining analytical ultracentrifugation (AUC), small angle x-ray scattering (SAXS), and constrained scattering modeling, we have analyzed the solution structures for a range of purified heparin and HS fragments. Solution structures of heparin and HS complexes with FH are likewise not known, and were determined. To date, our solution structures for the heparin complex with FH SCR-6/8 shows that FH SCR-6/8 forms large aggregates in the presence of heparin. Intact FH forms 5-15% of dimeric and higher oligomer structures in the absence of heparin. When heparin is titrated with FH, our AUC and X-ray data shows that heparin promotes further strong FH aggregation (up to 63%) as well as conformational changes in FH. These conformational changes and aggregation results provide the first molecular picture of how FH interacts with host cell surfaces and have a direct relevance for the sub-retinal pigmented endothelial deposits that form in Bruch's membrane during the development of AMD. SPR of immobilized heparin with full length FH gave K_d values of 1–3 μM, and weaker K_d values of 4–20 μM for the FH SCR-6/8 and FH SCR-16/20 fragments, confirming co-operativity between the two binding sites.

Acknowledgments

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A novel sialic acid-specific lectin from the mushroom *Hericium erinaceum*

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Sialoglycoconjugates have a variety of biological functions and involve the mediation and/or modulation of numerous biological phenomena [1]. Mushroom lectins harboring carbohydrate binding specificity are a powerful tool to detect sialoglycoconjugate with $\alpha(2,3)$ -, $\alpha(2,6)$ -, and $\alpha(2,8)$ -linkages [2]. However limited numbers of sialic acid-specific binding lectins are available to detect linkage of glycoconjugates [3]. In this study, a sialic acid-specific binding lectin was identified and characterized from the fruiting body extract of a mushroom *Hericium erinaceum*. The sialic acid binding lectin was purified by a combination of ion-exchange column, an immobilized fetuin column and gel filtration chromatography. The purified lectin was designed as HEL (*H. erinaceum* lectin). Tricine-PAGE, MALDI-TOF mass spectrometry, and N-terminal amino acid sequencing indicated that the native HEL has an identical form with a molecular weight of approximate 15 kDa. Isoelectric focusing of the lectin showed bands near pI 5.4. Hemagglutination assay displayed the agglutination activity of HEL was more effective against porcine erythrocyte rather than other animal red blood cells. When HEL was analyzed with glycan microarray, it was observed that the lectin binds to Neu5Ac, Neu5Gc and other sialic acid derivatives. Furthermore, HEL bound to $\alpha(2,3)$ -sialylated fetuin and not to asialofetuin. Thus, HEL will be a promising tool for detection of linkage-specific sialic acid in glycoconjugates.

Acknowledgements

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Enzymatic production of a natural solubilizer rubusoside using a thermostable lactase from *Thermus thermophilus*

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The major challenge with the design of oral dosage is its poor solubility and low permeability. Among the bioactive compounds obtained from plant sources, it has been discovered that some steviol glycosides such as stevioside (Ste), rebaudioside A, and rubusoside possess solubilizing properties. Rubusoside (RU) is a natural sweetener and a solubilizing agent with antiangiogenic and ant allergic properties. However, currently, its production is quite expensive, and therefore to find enzymes for more efficient conversion of Ste to Ru, thirty commercial enzymes were tested, having the mixed activities of pectinase, cellulases, hemicellulases, α -galactosidase, β -galactosidase and/or β -glucanase, along with a purified recombinant lactase. Among these, the recombinant lactase from *Thermus thermophilus* showed the highest Ru productivity. Therefore, it was chosen for immobilization on sodium alginate beads, and used to prepare Ru. Recombinant β -galactosidase of *Thermus thermophilus* (6 KU) was mixed with 20 mL of 3% (w/v) sodium alginate solution to give a final 300 U.mL⁻¹ of sodium alginate bead. The free enzyme was kept at 70°C and the thermo stability was compared with the immobilized enzyme. Immobilized lactase was prepared and used for the production of rubusoside; twelve reaction cycles were repeated with 95.4% of Ste hydrolysis and 49 g.L⁻¹ of Ru was produced. The optimum rubusoside synthesis yield was 86% at 200 g.L⁻¹, 1200 U lactase. The reaction mixture containing 20 mL of 1% (w/v) Ste solution in Tris-HCl buffers (40 mM, pH 7.0) and 10 mL alginate beads (300 U.mL⁻¹ beads) were incubated at 70°C in a water bath. After the enzymatic reaction had proceeded for 24 h. One hundred mg RU and 10 mg insoluble compounds were weighted and mixed at the ratio of 10:1 (w/w). One mL of absolute ethanol was added in a tightly sealed tube and mixed for 15 min. The mixture solution was centrifuged at 12,000 rpm for 10 min and the supernatant was transferred to another Eppendorf tube. The ethanol was evaporated. The purified 10% rubusoside solution showed increased water solubility of liquiritin from 0.98 mg.mL⁻¹ to 4.70 ± 0.12 mg.mL⁻¹ and 0 mg.mL⁻¹ to 3.42 ± 0.11 mg.mL⁻¹ in the case of teniposide. (Poster Presentation Abstract)

Aacknowledgement

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Production of rubusoside from stevioside by using a thermostable lactase from *Thermus thermophiles* and solubility enhancement of liquiritin and teniposide. *Enzyme Microb Technol* 2014 Oct 11;64-65:38-43. 2014.

Practical preparation of sugar 1-phosphates

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Sugar 1-phosphates are biochemically important compounds for precursors of NDP-sugars as well as substrates for phosphorylases. We here show the preparations of these compounds. Some of sugar 1-phosphates were produced by the phosphorolysis of oligosaccharides using phosphorylases and others were by phosphorylation of monosaccharides using anomeric kinases in the presence of an ATP-regeneration system. The sugar 1-phosphates produced were isolated by employing electro dialysis steps using two different membrane cassettes and finally crystallized as certain salts. We have prepared the following sugar 1-phosphates in more than gram-scale: β -D-glucose 1-phosphate (by maltose phosphorylase), α -D-mannose 1-phosphate, *N*-acetyl- α -D-glucosamine 1-phosphate, *N*-acetyl- α -D-galactosamine 1-phosphate (by *N*-acetylhexosamine 1-kinase), α -D-galactose 1-phosphate, α -D-galacturonic acid 1-phosphate, β -L-arabinose 1-phosphate (by galactokinase), β -L-fucose 1-phosphate, α -D-arabinose 1-phosphate (by fucokinase).

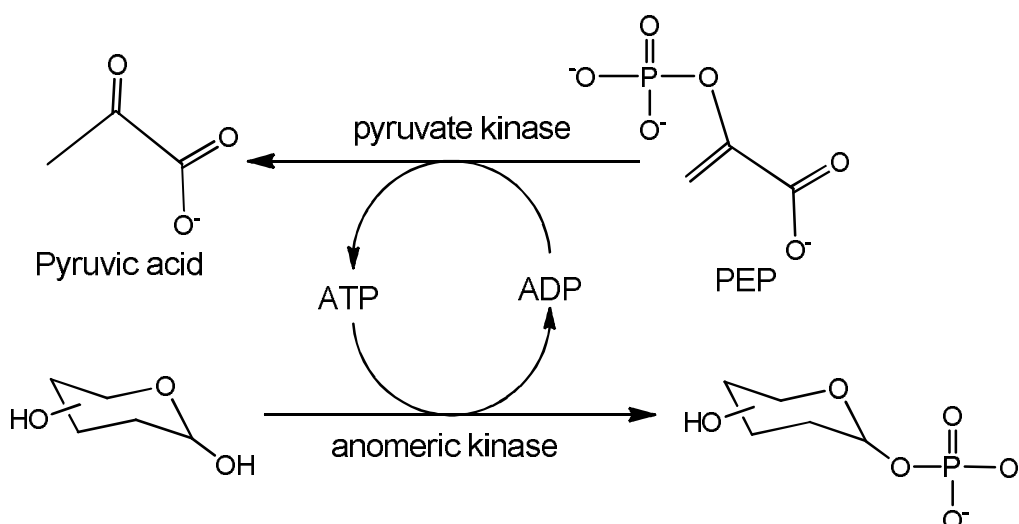


Fig 1. Anomeric phosphorylation by anomeric kinase with an ATP-regeneration system

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Structural and functional insights into the CBM50s of two plant GH18 chitinases

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Family 50 carbohydrate-binding modules (CBM50s) are also known as LysM domains. They are protein modules that are widely distributed in eucaryotes and procaryotes. Although CBM50s are found in various proteins, main chain structure, *i.e.* $\beta\alpha\alpha\beta$ fold, and ligand binding sites are well conserved. In the present study, we solved the crystal structure of two CBM50s of plant GH18 chitinases, *Pteris ryukyuensis* chitinase-A (PrChi-A) and *Equisetum arvense* chitinase-A (EaChi-A) at 1.8 and 2.5 Å resolution, respectively. In these two structures, two disulfide bonds were well conserved, suggesting that these bonds are important in the conformational stability of the modules (Fig 1). Interestingly, these bonds are not conserved in other CBM50s. The chitin binding sites of the CBM50s from PrChi-A and EaChi-A were estimated based on the NMR titration experiments. Based on the results obtained, we identified the binding sites located in a shallow groove formed by the loop between strand 1 and helix 1, the N-terminal part of helix 1, the C-terminal part of the helix 2, and the loop between helix 2 and strand 2. It should be noted that the dramatic line broadening of the NMR signal of the solvent exposed tyrosine residues (Y72 of PrChi-A and Y15 of EaChi-A) were observed upon ligand binding. To verify the involvement of these residues in chitin binding, binding affinity of the wild-type and Tyr mutants (Y72A, Y72F, Y72W) to chitohexaose was measured using isothermal titration calorimetry (ITC). Mutation of these residues to alanine resulted in K_a values which are about one order of magnitude lower than that of the wild-type, indicating the crucial role of aromatic residues in chitin binding.

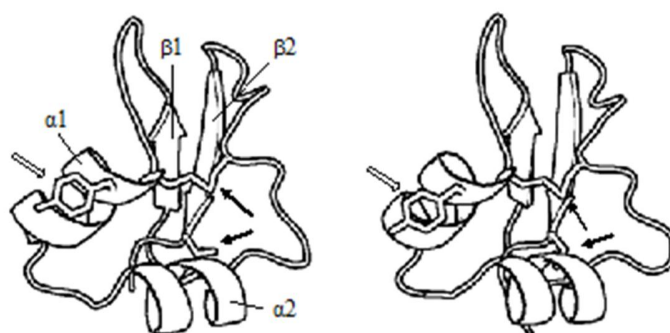


Fig 1. Crystal structures of two CBM50s from plant chitinases. Crystal structures of PrChi-A LysM2 (left, PDB; 4PXV) and EaChi-A LysM (right) are illustrated in ribbon models. White arrow: surface exposed tyrosine residue, black arrow: intra-domain disulfide bonds.

New glucuronoyl esterases for wood processing

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The development of new wood-based materials is of great interest to the forest industry. Wood tissue is composed of a complex biopolymer mixture containing cellulose, hemicellulose and lignin. Covalent bonds between lignin and different polysaccharides form closely associated structures known as lignin-carbohydrate complexes (LCCs). As a result, the successful extraction and separation of wood polymers poses a major challenge for materials biorefinery concepts. Enzymes that target lignin-carbohydrate (LC) bonds are especially useful for biorefinery applications as they can facilitate the isolation of individual wood components in combination with mild chemical treatments.

The main LCCs present in wood are believed to be esters, benzyl ethers and phenyl glycosides [1,2]. Glucuronoyl esterases (GEs) have been proposed to degrade ester bonds between glucuronic acids in xylans and lignin alcohols. GEs belong to the carbohydrate esterase (CE) 15 family and are present in the genomes of a wide range of fungi and bacteria.

The aims of our study were to characterize new GE enzymes, to investigate their capacity in disconnecting hemicellulose from lignin and to apply them in the extraction process. Selected candidate genes encoding novel GEs from a diverse range of filamentous fungi were produced in the eukaryotic enzyme production host *Pichia pastoris*. Purified enzymes were tested on model substrates as well as LCC fractions and their applicability in wood processing is investigated.

Acknowledgements

Research on glucuronoyl esterases at Chalmers is funded by the Knut and Alice Wallenberg Foundation. Selection of candidate genes was performed in the context of OPTIBIOCAT (FP7 KBBE. 2013.3.3-04), which is gratefully acknowledged.

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Comparison of transglycosylation abilities of two α -L-fucosidase isozymes from *Paenibacillus thiaminolyticus*

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α -L-Fucosylated oligosaccharides take part in a number of biological processes such as fertilization, cell adhesion, cell proliferation and processes of inflammation. They are also responsible for existence of blood group antigens. Changes in fucosylation of oligosaccharides were observed in association with several diseases, like cancer, rheumatoid arthritis and cystic fibrosis. Fucosylated oligosaccharides are used in pharmaceutical, cosmetics and food industry however their preparation via organic synthesis is a difficult and complicated process, which could be simplified by using enzymatic synthesis instead. Our previous research indicate, that α -L-fucosidase from *Paenibacillus thiaminolyticus* has great potential for artificial fucosylation [1], [2], [3]. There exist two isozymes of this protein. Both recombinant isozymes were produced, isolated and characterized. Preliminary test of substrate specificity was conducted using selected substrates. Ability to catalyze transglycosylation reactions of studied enzymes was tested and compared. Gained results indicate that isozyme 1 shows higher potential to catalyze transglycosylation reactions. Crystallographic experiments were also carried out on both isozymes.

Acknowledgements

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Variations in the substrate specificity of cellobiose dehydrogenase

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Cellobiose dehydrogenase (CDH) is an extracellular redox enzyme secreted by wood-rotting fungi during lignocellulose degradation. Recently it was shown that CDH can act as a reductase for lytic polysaccharide monooxygenases (LPMOs), which boosts the depolymerization of crystalline cellulose, hemicellulose or starch. CDH's unusual architecture comprises a catalytic flavodehydrogenase domain connected to a mobile cytochrome domain. This bipartite structure not only is unique for an extracellular protein but is also essential for the interaction with LPMOs and a key feature for its technical utilization in biosensors or the production of aldonic acids.

Data mining in available fungal genomes provided more than 300 putative *cdh* genes, which form three phylogenetic branches: Class I CDHs from basidiomycetes and class II or class III CDHs from ascomycetes. Recombinant production and characterization of more than 15 CDHs from different branches shows a correlation to the phylogenetic classification. Differences are not only of structural, but mostly of catalytic nature. Class I CDHs generally show a preference for the natural substrate cellobiose, while substrate spectra of class II CDHs extend to carbohydrates found in hemicellulose such as mannopentaose, xylo-oligosaccharides or maltose and malto-oligosaccharides. In addition, some class II CDHs show neutral or alkaline pH optima not observed for the classically known (acidic) class I CDHs. These abilities clearly distinguish class II CDHs from the well-studied class of basidiomycetous CDHs and are an important step towards the development of CDH-based biocatalytic processes and biosensors. The enigmatic class III CDHs, which have not been characterized so far, show a dramatically altered active site architecture. First results of their expression and characterization will be presented. Taken together, these varying substrate specificities may help to understand the interaction with LPMOs and are of interest for the production of various aldonic acids.

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The first transglycosidase derived from a GH20 β -*N*-acetylhexosaminidase

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The β -*N*-acetylhexosaminidase (EC 3.2.1.52, CAZy GH20) from the filamentous fungus *Talaromyces flavus* has been shown to possess unique substrate flexibility and remarkable synthetic ability. However, the yields of the transglycosylation reactions are significantly lowered by the undesired hydrolysis of the glycosidic bonds of the substrate and product. To overcome this problem, **mutant variants of *T. flavus* β -*N*-acetylhexosaminidase** were designed based on a computational model of the active site of this enzyme, aiming at diminishing its hydrolytic activity and retaining transglycosylation activity at once, e. g. at the preparation of a transglycosidase [1].

Three mutants of β -*N*-acetylhexosaminidase were produced in the *Pichia pastoris* expression system in high yields, purified from the culture media and characterized. All of the **mutant variants featured strongly (ca 200-fold) reduced hydrolytic activity** while maintaining high transglycosylation activity reaching extraordinary conversion rates. Reaction with *p*NP-GlcNAc as a substrate yielded mixtures of ***p*NP-chitooligosaccharides containing up to 8 *N*-acetylglucosamine units**. Also natural substrates - chitooligomers (DP 2 - 3) - were rearranged by these transglycosidases to **rare oligomers with higher degrees of polymerization (DP 4 – 8)**. GlcNAc-linker-*t*Boc was extended to *t*Boc-chitooligosaccharides enabling thus simple creation of multivalent molecules. This enzyme has a potential to attach β -GlcNAc units to a variety of other acceptors analogously as the wild enzyme but in a considerably higher yield. In summary, we have designed and prepared three variants of a novel transglycosidase derived from β -*N*-acetylhexosaminidase from *T. flavus*, able to catalyze the synthesis of valuable *N*-acetylchitooligomers without unfavourable hydrolysis.

This is **the first report on a transglycosidase** stemming from an *exo*-glycosidase employing the **substrate-assisted catalytic mechanism** *via* the oxazoline reaction intermediate.

Acknowledgements: The financial support by the project from Czech Academy of Sciences M200201204 and by the Czech Science Foundation Grant 15-02578J is gratefully acknowledged.

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Carbohydrate composition in spruce bark

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Norway spruce (*Picea abies*) bark is an abundant lignocellulosic feedstock having potential as a source of biofuels and biochemicals based on phenolics and carbohydrates. This poster summarizes the overall carbohydrate profile of spruce bark, discusses the solubility and extractability of different carbohydrate fractions, and compares the results to spruce wood carbohydrates based on published¹ and unpublished data.

Spruce bark dry matter was found to contain circa 20% cellulose, 21-24% non-cellulosic neutral carbohydrates and 7-9% acidic carbohydrates. Cellulose content of bark was significantly lower than cellulose content of spruce wood (35%). Non-cellulosic neutral carbohydrates were mainly composed of non-cellulosic glucose (8-12% of bark dry matter), which can be present in bark as a part of non-cellulosic polysaccharides, as free glucose or sucrose, or as glycosides, e.g. as a part of stilbene glycosides. According to glycome profiling analysis carried out with an extensive set of glycan-directed monoclonal antibodies, spruce bark contained xyloglucan, which is one source for non-cellulosic glucose. Starch and β -glucan contents were found to be below 1%. Hot water extracts prepared from bark collected from logs felled in the winter contained free glucose that composed up to 2% of bark dry matter, whereas corresponding samples from bark collected from logs felled in the summer contained very low amounts of free glucose. Apparently the season affected the amount and type of non-cellulosic glucose and glucan in bark. Stilbene glycosides such as isorhapontin and astringin are abundant in spruce bark² and there is some indication that glucose may be bound to condensed tannin units as well³. Content of mannose-containing polysaccharides in bark was found to be much lower (2-3%) compared to wood (12-13%), but pectin content in bark was significantly high, up to 7% of bark dry matter based on the content of polygalacturonic acid. Glycome profiling showed also the presence of arabinogalactans in bark.

Acknowledgements The research leading to these results has received funding from the WoodWisdom-Net Research Program which is a transnational R&D program jointly funded by national funding organizations within the framework of the ERA-NET WoodWisdom-Net 2.

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Enzymatic synthesis of functional linear isomaltomegalosaccharide by *Gluconobacter oxydans* dextran dextrinase

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By definition, the megalosaccharide has DP between 10 and 100 (Fig. 1A). Linear isomaltomegalosaccharide (L-IMS), which is linear-type α -(1 \rightarrow 6)-glucosyl megalosaccharide, possesses beneficial functions of bioavailability of flavonoid glycoside (Fig. 1B) [1-3]. Dextran dextrinase from *Gluconobacter oxydans* (DDase; EC 2.4.1.2), a secreted protein of *G. oxydans* [4], produced dextran from maltooligosaccharides by its transglycosylation activity, whereas C-terminal deletion mutant (Δ 382C) could efficiently produce L-IMS (Fig. 1C). The yield of L-IMS against substrate by DDase and Δ 382C was 21% and 41% (w/w), respectively. C-terminal truncation study revealed that the deletion of B region increased the yield of L-IMS. Mutational experiment found that the substitution of Asn916 in B region with Ala increased L-IMS productivity. Our study revealed that B region of DDase controls saccharides length of product.

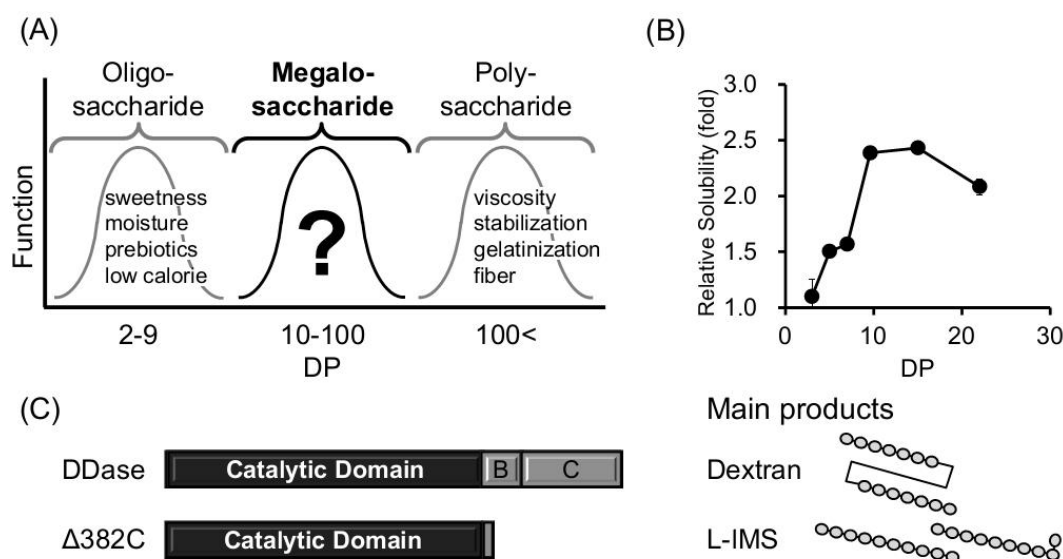


Fig 1. Definition of megalosaccharide, and function and production of L-IMS. (A), Size-dependent functions of saccharides; (B), Effect of DP on quercetin-3-O- β -D-glucoside (Q3G) solubility. Relative solubility of 1.0 is the Q3G-solubility in water; (C), Schematic presentation of the enzymes and their main end products.

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Genomics, systematics and proteomics of the wood-decomposing white rot Basidiomycota Polypore species *Phlebia radiata*

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The systematically incoherent genus *Phlebia* consists of white rot species which produce a variety of lignocellulose-degrading CAZymes (carbohydrate-acting enzymes) and oxidoreductases. Due to the capability for efficient conversion of wood, plant biomass and harmful organic compounds, the type species of this genus, *P. radiata*, was selected for genome and transcriptome sequencing. The nuclear and mitochondrial genomes of Finnish wild-type heterokaryotic isolate *P. radiata* 79 (FBCC43) were assembled by using PacBio RSII and Roche 454 FLX Titanium sequencing platforms. *P. radiata* mitochondrial genome is the second largest (over 156 kbp) sequenced and gene annotated for fungi [1]. Nuclear genome sequencing produced 4.34 Gbp of data with mean read length of 8095 bp. HGAP3 pipeline assembly produced 127 contigs with 37 longest containing more than 90% of the assembly (contig N50 1.984 Mbp), summing up to 40.92 Mbp haploid genome size.

In addition to genome sequencing, our aim was to provide an assessment of protein coding genes and proteins that function in core fungal metabolism, degradation of plant cell wall lignocelluloses (PCWL) and oxidation of lignin-like and polluting compounds. The fungus was cultivated on spruce wood, and extraction of RNA and proteome were performed weekly for parallel cultures. Extracellular CAZy enzyme activities were followed in liquid and wood cultivations. Secreted wood-decay enzymes were constantly produced when the fungus was growing on spruce wood. Lignin peroxidases (LiP) were dominant oxidoreductases in the proteome. Besides LiPs, other lignin-modifying class II peroxidases (manganese peroxidases), many cellulolytic CAZymes, and auxiliary hydrogen peroxide producing enzymes were the most identified proteins. The results show that *P. radiata* has a functional and versatile wood and plant biomass degrading enzyme machinery.

Acknowledgements The study was supported by the Academy of Finland Ox-Red research project, grant 113833, and University of Helsinki Doctoral Programme in Microbiology and Biotechnology.

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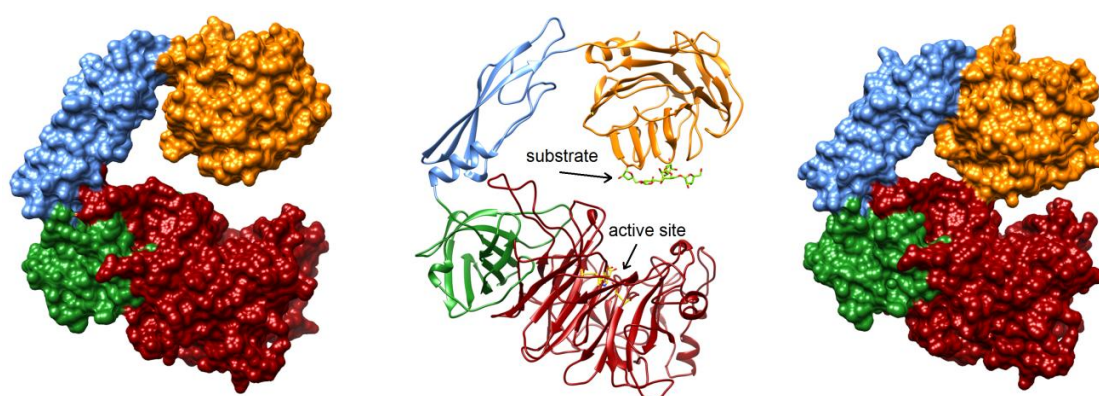
A unique multi-domain extracellular GH43 arabinanase determined in different conformational states

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α -L-arabinanases are key enzymes in the breakdown of arabinan, one of the main polysaccharides in the plant cell-wall, and hence present a wide range of important potential biotechnological applications. *GsAbn43A* is an extracellular α -L-arabinanase from the thermophilic Gram-positive bacterium *Geobacillus stearothermophilus*-T6, shown to degrade efficiently linear and naturally-branched arabinan. The enzyme belongs to family GH43, contains 848 amino-acid residues, and possesses relatively low sequence identity to related arabinanases. The 3D structure of *GsAbn43A* has recently been determined by X-ray crystallography, revealing a unique and novel multi-domain architecture, the largest reported so far in the GH43 family. The enzyme is built of four different domains, arranged in a pincer-like structure. The catalytic domain (shown in red in the figures below) corresponds to the five-bladed β -propeller fold observed in GH43 enzymes. The second domain (green) has also been seen before in some homologous two-domain arabinanases, but the third (blue) and fourth (orange) domains are unique to *GsAbn43A*. Interestingly, two different conformational states have been determined for the enzyme, a "closed" state (right figure) and an "open" state (left), differentiated by a ~ 12 Å movement in location of the fourth domain. Substrate-binding structural experiments demonstrate, surprisingly, that an arabinopentaose substrate binds to a dedicated site in the fourth domain, rather than to the expected catalytic domain and active site (central figure). These findings, together with complementary ITC and kinetics experiments, suggest a novel catalytic mechanism for the arabinan degradation action of *GsAbn43A*.



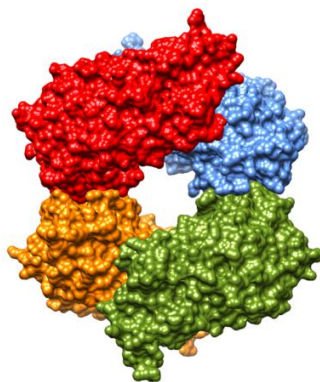
Structural analysis of Abp, a GH27 β -L-arabinopyranosidase from *Geobacillus stearothermophilus*

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L-arabinose sugar residues are relatively abundant in plants, found mainly in arabinan polysaccharides and other arabinose-containing polysaccharides, such as arabinoxylans and pectic arabinogalactans. The majority of the arabinose units in plants are present in the furanose form and only a small fraction are present in the arabinopyranose form. We have recently characterized the L-arabinan utilization system in *Geobacillus stearothermophilus* T-6, a Gram-positive thermophilic soil bacterium, where one of the key enzymes was found to be an intracellular β -L-arabinopyranosidase (Abp). The detailed three-dimensional structure of Abp-WT has been determined by X-ray crystallography to 2.28Å resolution, as well as the structure of one of its catalytic mutants (Abp-D197A) in complex with a β -L-arabinose molecule (2.19Å resolution). These structures demonstrate that the 3D structure of the Abp monomer generally correlates with the general fold observed for GH27 proteins, consisting of two main domains, an N-terminal TIM-barrel domain and a C-terminal all- β domain. The two catalytic residues are located in the TIM-barrel domain, such that their carboxylic functional groups are about 5.9 Å from each other, consistent with a retaining mechanism. An isoleucine residue (Ile67), located at a key position in the active site, is shown to play a critical role in the substrate specificity of Abp, providing structural basis for the high preference of the enzyme toward arabinopyranoside over galactopyranoside substrates. The crystal structure demonstrates that Abp is a tetramer composed of two "open pincer" dimers, clamped around each other to form a central cavity (Figure). The four active sites of Abp are situated at the inner surface of this cavity, all opened into the central space of the cavity. The biological relevance of this tetrameric structure is supported by independent results obtained from gel-filtration, DLS and SAXS experiments. These data and their comparison to the structural data of related GH27 enzymes are used for a more general discussion concerning structure-selectivity aspects in this GH family.



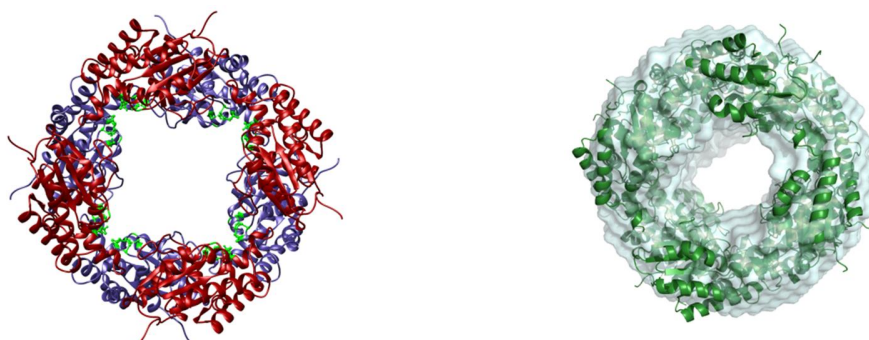
A unique octameric structure of an acetyl-xylan esterase

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Geobacillus stearothermophilus T-6 is a thermophilic soil bacterium that possesses an extensive and highly regulated hemicellulolytic system, allowing the bacterium to efficiently degrade high molecular weight polysaccharides such as xylan, arabinan and galactan. As part of the xylan degradation system, the bacterium uses a number of side-chain cleaving enzymes, one of which is Axe2, a 219 amino-acids intracellular serine acetylxylan-esterase that removes acetyl side-groups from xylo-oligosaccharides. Axe2 belongs to the lipase GDSL family and represents a new family of carbohydrate esterases. The detailed three-dimensional structure of Axe2 has recently been determined by X-ray crystallography demonstrating it is arranged as a "doughnut-shaped" homo-octamer (left figure). Gel-filtration, TEM and SAXS (right figure) experiments all confirm the octameric torus structure of Axe2 is indeed the biologically relevant structure in solution. The Axe2 octamer is built of two staggered tetrameric rings, where all eight active sites face the relatively wide internal cavity, and is held together by three specific types of monomer–monomer interfaces, each of which involves a number of intermolecular interactions. A series of rational noncatalytic, site-specific mutations were made in order to further examine the functional significance of this oligomerization, some of which lead to a different dimeric form of Axe2 showing a significant reduction in catalytic activity. The structure of one of these mutants, Axe2-Y184F-W190P, has recently been solved by X-ray crystallography, and offers a possible explanation for the functional origin of this unique oligomeric structure.



Characterization of a Chitin Utilization Locus from *Flavobacterium johnsoniae*

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The bacteria belonging to the Bacteroidetes phylum are well known for their polysaccharide degradation capabilities, with the most well studied species reside in the gastrointestinal tract of animals. The enzymes responsible for the degradation of complex glycans are often parts of distinct gene clusters named Polysaccharide Utilization Loci (PULs). PULs contain outer membrane sugar binding proteins and transporters, as well as sugar sensors and regulatory elements and a varying number of degradative enzymes. Thus far, a multitude of PULs have been identified in Bacteroidetes species from varying habitats, mainly targeting non-crystalline polysaccharides (e.g. hemicelluloses, pectins and host glycans) as well as different types of starch.

A PUL responsible for the degradation of chitin has been identified in the aerobic soil bacterium *Flavobacterium johnsoniae* [1]. Chitin is a crystalline polysaccharide found in shells of arthropods and cell walls of fungi, and is the second most abundant carbohydrate on earth after cellulose. In the current study, the chitin utilization locus (ChiUL), consisting of two SusC/D pairs, a sensor-regulator pair, an inner membrane transporter, and four enzymes, is characterized in detail. The main chitinase of the ChiUL, ChiA, is secreted into the environment via the newly discovered type IX secretion system, therefore not conforming to the typical PUL arrangement, where polysaccharide-active enzymes are attached to the outer membrane. The ChiUL contains three chitinase (GH18) domains, two in ChiA and one in ChiB. These three GH18 enzymes are not highly homologous and display different activities on their substrates, which include both crystalline α - and β -chitin and chitooligosaccharides. ChiB is predicted to be located in the periplasm together with a GH20 enzyme, facilitating the conversion of imported oligosaccharides into GlcNAc.

In addition to the enzymology, gene knock-out studies have shown how both the SusC/D pairs, the sensor-regulator pair, and the inner membrane transporter are indispensable for growth on chitin. However, ChiA is the only singular enzyme gene essential for growth. Further, isothermal calorimetry has been performed to evaluate the binding affinities of the SusD-like outer membrane proteins for chitooligosaccharides, revealing distinct preferences for sugar length.

The study of the ChiUL from *F. johnsoniae* enables us to understand how highly recalcitrant crystalline polysaccharides can be utilized by the sophisticated carbohydrate degradation systems of the Bacteroidetes.

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Recombinant production of an exopolysaccharide of interest for health industry

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Polysaccharides have many applications in the health and cosmetic industries. These molecules are extracted from plants, algae and animals. Microorganisms are also able to produce innovative exopolysaccharides (EPS). This way of production exhibits a great interest for the industrial demand. On one hand, the microbial diversity allows opportunities to discover high-value new molecules; on the other hand, it provides a production independent of climatic or seasonal changes, easy to optimize and control as well as an easier and safer extraction process. The marine bacteria *Vibrio diabolicus* [1] produces an EPS composed of uronic acid, *N*-acetyl-glucosamine and *N*-acetyl-galactosamine, the EPS HE800 [2]. Its chemical structure is similar to the hyaluronic acid one, an EPS widely used in human health. The EPS HE800 has already shown efficiency on bone regeneration [3]. To improve the EPS HE800 applications and production, an accurate knowledge of its biosynthesis is required. The cluster responsible for the production of the EPS HE800 has been identified. Cloning and expression in *Escherichia coli*, a common heterologous host, is studied in order to improve the control of the production and later plan the production of targeted functionalised molecules. Data on the EPS HE800 recombinant production will be presented. These would include EPS production tests, molecular mechanisms of the polysaccharide biosynthesis, transcriptional analysis of the main genes responsible for the production of this EPS. This study provides a better understanding of the biosynthesis of a new molecule of interest in health industry, in order to improve and control its production.

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Exploring complex glycan utilization machinery of *Roseburia* spp. implicated in inflammatory and metabolic disorders

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The human gastrointestinal tract is colonized by a dense, dynamic and complex microbiota. Humans are unable to degrade most dietary glycans, which instead are metabolised by different gut bacteria in the lower part of the gastrointestinal tract. The metabolism of dietary complex polysaccharides by distinct gut microbiota taxa has a pronounced effect on human health and physiology, which is at least partly associated with the specific short chain fatty acid (SCFA) fermentation products of glycans that vary between different taxa.

To date, a relatively narrow taxonomic range of bacteria involving mainly probiotic strains from *Lactobacillus* and *Bifidobacterium* genera have been explored for their health promoting properties. The therapeutic potential of other gut commensals remains ill-explored. Recent evidence correlates decreased abundance of gut commensal bacteria from the *Roseburia* genus to inflammatory and metabolic disorders such as Crohn's disease and type 2 diabetes (1-2). Furthermore, *Roseburia* spp. are reported to be primary degraders of complex polysaccharides including starch and inulin (3), which are fermented to butyrate. Butyrate production plays a role in the maintenance of colonic homeostasis, and increased concentrations of this glycan fermentation product in the colon are associated with anti-inflammatory and anti-tumorigenic effects.

A more detailed investigation of the glycan metabolic repertoire of *Roseburia* spp. is necessary to reveal features underpinning its specialization and ultimately to explore its potential in therapeutic interventions targeting inflammatory and metabolic disorders. This project addresses the gap in our knowledge on the above aspects by characterization of selected glycan active proteins from the taxon. The latest results will be presented and discussed.

Acknowledgements

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Structural and functional insights on the glycoside hydrolases involved in the metabolism of xylooligo- and arabinooligosaccharides in lactic acid bacteria

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Arabino- and xylo-oligosaccharides (AOS and XOS) have gained increased interest as prebiotics during the last years. AOS and XOS can be produced from major fractions of biomass including agricultural by-products and other low cost raw materials. *Weissella* and *Lactobacillus* can be considered representative genus of lactic acid bacteria. Strains of *Weissella cibaria/confusa* have recently been proposed as potential probiotics based on growth and adsorption to human cells {Lee, 2012 #24}, while *Lactobacillus* strains have been broadly used as models for studies of probiotics. The metabolism of the AOS/XOS is still poorly understood in lactic acid bacteria. Hydrolysis of AOS/XOS is one of the first steps in the metabolism. The main hydrolases involved in converting AOS and XOS to monosugars are arabinosidases and xylosidases. In lactic acid bacteria, these enzymes are found in families GH8, GH43 and GH51 (www.cazy.org), where GH43 seems most broadly present in lactic acid bacteria.

We have done a comparative study of the structure/function of arabinofuranosidases and xylosidases from GH43 in novel strains from *Weissella* and in *L. brevis* DSM 1269. Previously, both activities have shown preference for short natural substrates (di- and tri-saccharides) in enzymes cloned from *L. brevis* DSMZ 20054 {Michlmayr, 2013 #74}, and similar preferences were shown for enzymes cloned from *L. brevis* DSMZ 1269 and from *Weissella* strains. Kinetics studies also suggest that *Weissella* enzymes are more active than the corresponding candidates from *L. brevis*. All studied enzymes are intracellular, lacking signal peptides. Structurally, arabinofuranosidases and xylosidases share a five-bladed β -propeller fold in the catalytic domain, while the studied xylosidases have an additional domain with β -sandwich fold in the C-terminal end. The role of this additional domain is still unknown, however, its mutational deletion have shown it to be fundamental for the xylosidase activity. Crystallographic studies and size exclusion chromatography analysis are consistent with dimerization in native arabinofuranosidases for *Weissella* and *L. brevis* DSM 1269. However, although the xylosidases are present mostly as tetrametric structures, a single mutation in a superficial loop of the catalytic domain could affect the stability of the oligomers. These structural and functional implications will be discussed.

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β -D-galactosidase/fucosidase from *Paenibacillus thiaminolyticus* and its transglycosylation properties and immobilization

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β -D-Galactosidases are enzymes which have been for many years applied in biotechnological processes for different purposes. Their ability to catalyze transgalactosylation reactions enables the production of galactooligosaccharides, beneficially affecting intestinal microbiota or galactosylated molecules with the potential for pharmaceutical industry. Some of these widespread enzymes are known to often display not only β -D-galactosidase activity, but also the β -D-fucosidase, β -D-glucosidase, or α -L-arabinosidase activity. β -D-Galactosidase with β -D-fucosidase activity from the bacterial strain *Paenibacillus thiaminolyticus* was produced in the form of histidine-tagged protein, and purified using affinity chromatography. Kinetic parameters of β -D-galactosidase were determined with *p*-nitrophenyl β -D-galactopyranoside, *p*-nitrophenyl β -D-fucopyranoside and also with *p*-nitrophenyl β -D-glucopyranoside and lactose. The ability to catalyze transfucosylation reactions with varied acceptors was tested. The enzyme catalysed also the transfer of fucosyl moiety to many different acceptor molecules, and some products were confirmed by mass spectrometry and NMR. Also the prebiotic effect of fucosylated saccharides was tested. Further we were concerned to immobilization of this enzyme by entrapping on the surface of the synthetic lipid droplets, and as a lipid coated enzyme. Immobilized β -D-galactosidase was used to catalyze transgalactosylation reactions in the two-phase organic-aqueous systems. This system is suitable for glycosylation of substances poorly soluble in water.

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Cultivation strategies for Chitinase Expression in *Chitinibacter tainanensis*

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Chitin, a kind of polysaccharide, exists abundantly on the earth. Chitin are the major components of most fungal cell walls, insect exoskeletons and the shells of crustaceans. They are also the main components of the cell wall of prokaryotes. Chitin and its derivatives, such as oligochitin, chitosan and N-acetyl glucosamine (NAG), are utilized versatile and ubiquitously. They are applied on industrial, environmental, agricultural and biomedical fields.

Recently, *Chitinibacter tainanensis* with NAG as final unique product of chitin degrading was isolated from the soil in Southern Taiwan. Chitin is degraded with the fermentate of *C. tainanensis*. The yield was 0.75 g/g - 0.98 g/g depending on the substrates. In the work, various conditions of medium were applied during inoculation and fermentation. The morphologies of bacteria and enzyme activity were examined for optimization..



Fig 1. The morphology of *Chitinibacter tainanensis*.

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From glycoside hydrolase to transglycosidase through protein and reaction engineering

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If devoid of their dominant hydrolytic reactivity, glycoside hydrolases would be excellent tools for glycosylation. They are characterized by robustness, absolute stereoselectivity and accept a wide variety of acceptors. Starting from a particularly stable β -glucosidase originating from the thermophile *Thermotoga neapolitana* and belonging to the well-studied glycoside hydrolase family 1, we have attempted to understand the factors governing the predisposition for hydrolysis or transglycosylation. With proper immobilization, low hydration decreased the unwanted hydrolytic reactivity of the glycosidase [1]. However, already at moderate hydration, the activity of the enzyme was reduced significantly. A more successful approach was protein engineering, where a single amino acid replacement improved the yield of our models target compound, hexyl- β -D-glucoside, from 17% to 58% [2]. Together with the observation that hydrolysis is more pH dependent than transglycosylation, we can limit the hydrolysis of the enzyme to an almost undetectable level, by running the reactions at high pH (Fig 1).

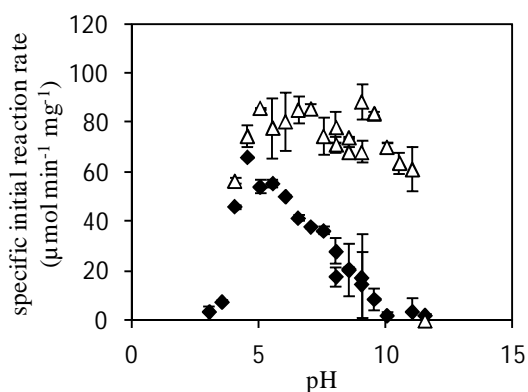


Fig 1. pH dependence of hydrolysis (◆) and transglycosylation (△) of *TnBg11A* mutant N220F. Error bars are 1σ .

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The impact of polysaccharide chemistry on the regioselectivity of AnAXE from *Aspergillus nidulans*

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Acetyl xylan esterases (AXEs) targeting polymeric or oligomeric substrates have been classified into 8 CE families, namely CE1-7, and CE12. AXEs from different CE families can differ in terms of their regioselectivity towards acetylated xylan¹⁻⁴. Briefly, characterizations to date indicate that esterases from families CE1, CE5 and CE6 can deacetylate mono- or di-acetylated methyl β -xylopyranoside², whereas family CE4 esterase target 2- and 3-monoacetylated xylopyranosyl residues⁵. Some AXEs are also active on cellulose acetate, a synthetic cellulose derivative, and show a distinct regioselective mode of action dependent on CE family^{6,7}. For example, CE1 esterases cleave substituents from the C2- and C3-positions of cellulose acetate. By comparison, the action of CE4 and CE5 esterases on cellulose acetate is restricted towards C3 and C2 substituents, respectively⁶. In addition to acetylated xylan and cellulose acetate, AXEs were previously shown to deacetylate native preparations of galactoglucomannan⁸. However, the regioselectivity of AXEs towards this substrate is unclear. In the current study, we investigate the impact of polysaccharide backbone and branching chemistry on the regioselectivity of a CE1 esterase from *Aspergillus nidulans*, namely AnAXE. In particular, acetylated forms of glucuronoxylan, galactoglucomannan, and cellulose were used in the analysis. Accessory enzymes, including a family GH115 α -glucuronidase and family GH27 α -galactosidase, were also used to distinguish impacts of backbone and branching substituents. Moreover, potential synergism between these debranching enzymes was evaluated.

Acknowledgements

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Plant biomass degrading potential of a new *Penicillium* species, *Penicillium subrubescens*

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A recently identified *Penicillium* species, *P. subrubescens* (Mansouri et al. 2013), was evaluated for its ability to hydrolyse plant biomass. Growth on various plant biomass related substrates demonstrated the capacity of this species to degrade all the main polysaccharides present in plant biomass as well as metabolise all their monomeric components. The ability to degrade broad range of carbohydrates suggests a high potential in plant biomass saccharification.

To evaluate this in more detail, *P. subrubescens* was grown on wheat bran and sugar beet pulp and a set of extracellular enzyme activities were analyzed from culture liquids. Also the ability to saccharify wheat bran and sugar beet pulp was determined. Compared to *P. chrysogenum*, *P. subrubescens* produced higher levels of β -glucosidase, endoglucanase, endoxylanase and cellobiohydrolase.

Enzyme mixtures produced on wheat bran by *P. subrubescens* were more efficient in saccharification of wheat bran compared to enzymes produced on sugar beet pulp cultures. The opposite result was observed for saccharification of sugar beet pulp. This demonstrates that *P. subrubescens* produces enzyme mixtures that are closely tailored to the available substrate, suggesting the presence of a fine-tuned regulatory system that controls the production of these enzymes.

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Genome and in-lab analysis of cold-tolerant xylanolytic *Paenibacillus* spp isolated from low level radioactive waste repository

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Paenibacillus spp are Gram-positive facultatively anaerobic bacteria related to *Bacilli*. They have been isolated from various environments including rhizosphere, hot springs, Antarctic and intestine of insects. For biotechnology, they have been studied for e.g. use as biofertilizers, production of antibiotics and plant biomass degrading enzymes. Here, several bacterial strains belonging to the genus *Paenibacillus* were isolated from low level radioactive repository experiment (Gas generation experiment (GGE), 1) operated by Teollisuuden Voima Oyj in Olkiluoto, Finland. The strains exhibit xylanolytic growth at 5-40°C and pH 5-9 and produce soluble and cell bound enzymes that hydrolyse polymeric xylan and *p*-nitrophenyl β-D-xylopyranoside. Taxonomically these paenibacilli are related to *P. borealis*, *P. odorifer*, *P. glucanolyticus* and *P. thaichungensis*. Genomic DNA of four strains related to *P. borealis*, *P. odorifer* and *P. thaichungensis* was sequenced with Illumina HiSeq followed by de novo assembly and in the case of strains related to both *P. borealis* and *P. odorifer*, mapping to available reference genomes. Genomic sequences are being explored for enzymes related to conversion of xylan and other carbohydrates.

Acknowledgements

Liisa Heikinheimo, Jenni Sauramo and Tuire Haavisto from Teollisuuden Voima Oyj (Finland) are thanked for provision of samples from GGE. Birgit Hillebrandt-Chellaoui and Helena Hakuli are thanked for technical assistance.

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Up-scaling of the synthetic procedure for preparation of oligosaccharide adjuvant for allergen immunotherapy

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Our group recently reported the synthesis and characterization of a new oligomannoside derived glycocluster (Fig. 1).¹ This compound has demonstrated considerable suppressive effects on the Th2-type allergic inflammatory response both in vitro and in vivo. In order to evaluate the possibility of bringing this product to clinical trials, a reliable procedure for up-scaling of its synthesis needs to be developed. In the present work, optimization of gram scale synthetic procedures was performed, including substitution of chromatographic purifications with crystallizations and extractions.

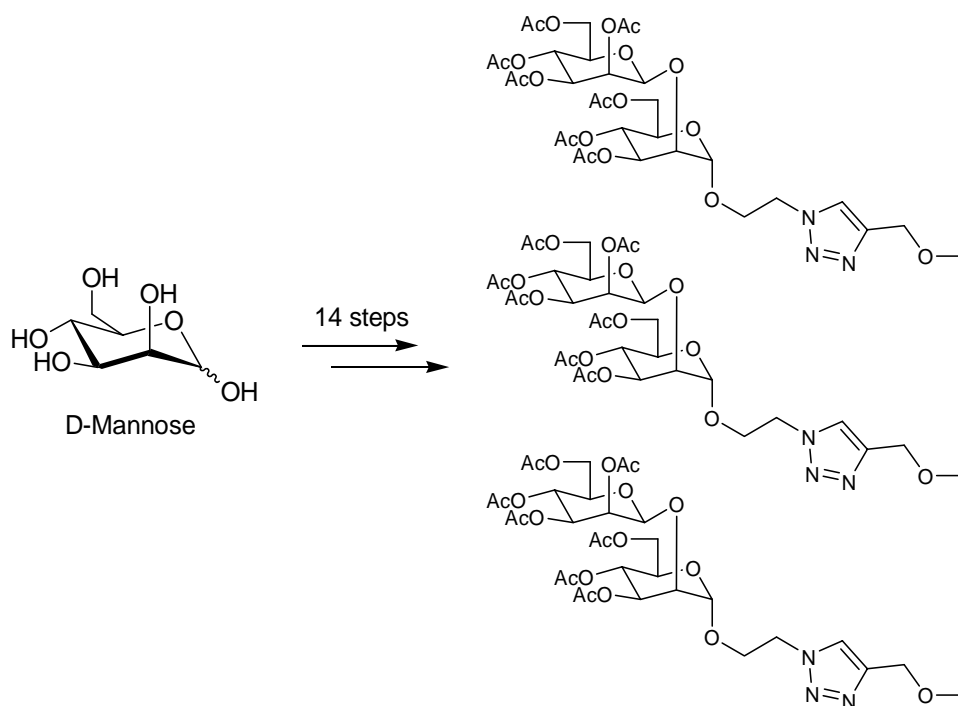


Fig 1. The target molecule

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Beechwood xylan for the measurement of *endo*-1,4- β -D-xylanase

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endo-1,4- β -Xylanase (β -xylanase) is most commonly assayed using birchwood xylan with measurement of reducing sugar using the DNSA assay procedure. Recently, the supply of birchwood xylan ceased. Unfortunately, the only other commercially available xylan, beechwood xylan, is not sufficiently pure for β -xylanase assays due to unacceptable high reducing sugar level leading to high background colour in the assay. In the measurement of *endo*-1,4- β -glucanase (cellulase) activity with the DNSA reducing sugar procedure, cellobiose is used as the standard instead of glucose. The reason for this is that under the highly alkaline conditions of this assay, some cello-oligosaccharides are chemically hydrolysed during incubation with DNSA, giving inflated hydrolysis values. Using cellobiose as the standard in place of glucose compensates for this. In β -xylanase assays, xylose has been routinely used as the standard because suitably pure xylobiose has not been readily available.

In the current study, we have critically compared birchwood xylan, beechwood xylan and wheat flour arabinoxylan for the measurement of β -xylanase using both the DNSA and the Nelson/Somogyi reducing sugar procedures, and have compared these assays to those using dyed arabinoxylan substrates. Standard curves for xylose and xylobiose in the presence of either the two xylans or arabinoxylan were not linear with the DNSA procedure, but were with the Nelson/Somogyi procedure. Also, with the DNSA procedure the colour response from xylobiose was approximately 140 % of that of an equimolar concentration of xylose. This, of course, has implications on the determined β -xylanase activity. Also, there is a difference in the sensitivity of the three substrates in the assay of β -xylanase. Each of these challenges in the measurement of β -xylanase will be discussed.

Novel substrates for the measurement of pullulanase

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Specific and highly sensitive chromogenic and fluorogenic substrate mixtures have been prepared for the measurement of limit-dextrinase and pullulanase activity and assays employing these substrates have been developed. These substrate mixtures contain thermostable α - and β -glucosidases and either 4,6-*O*-benzylidene-2-chloro-4-nitrophenyl- β -maltotriosyl (1-6) α -maltotriose (BzCNPG3G3) [I] as a chromogenic substrate, or 4,6-*O*-benzylidene-methylumbelliferyl- β -maltotriosyl (1-6) α -maltotriose (BzMUG3G3) [II] as a fluorogenic substrate. Hydrolysis of substrates [I] and [II] by *exo*-acting enzymes such as amyloglucosidase, β -amylase and α -glucosidase is prevented by the presence of the 4,6-*O*-benzylidene group on the non-reducing end D-glucosyl residue. The substrates also are not hydrolysed by any α -amylases studied (e.g. *A. niger*, porcine pancreas or bacterial) and are resistant to hydrolysis by *Pseudomonas* sp. isoamylase. On hydrolysis by pullulanase, the 2-chloro-4-nitrophenyl- β -linked maltotrioside is immediately hydrolysed to D-glucose and 2-chloro-4-nitrophenol (or methylumbelliferone). The reaction is terminated by the addition of a weak alkaline solution leading to phenolate ions in solution whose concentration can be determined using either spectrophotometry or fluorimetric analysis. The assay procedure is simple to use, specific, accurate, robust and readily adapted to automation. It should find widespread application in the screening of microbial preparations for this enzyme and also in the routine analysis of industrial pullulanase preparations and of plant limit-dextrinase.

Residue L940 has a crucial role in the specificity of the glucansucrase GTF180 of *Lactobacillus reuteri* 180

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Highly conserved GH70 family glucansucrases differ in catalyzing the synthesis of α -glucans with different structures from sucrose. The structural determinants of glucansucrase specificity have remained unclear. Residue L940 in domain B of GTF180, the glucansucrase of the probiotic bacterium *Lactobacillus reuteri* 180, was shown to vary in different glucansucrases and is close to acceptor binding site +1 in the crystal structure¹. Here, we show that mutations in L940 of wild-type GTF180- Δ N all caused an increased percentage of (α 1 \rightarrow 6) linkages and a decreased percentage of (α 1 \rightarrow 3) linkages in the products². α -Glucans with potential different physico-chemical properties [containing 67% to 100% of (α 1 \rightarrow 6) linkages] are produced by GTF180 and its L940 mutants. Mutant L940W was unable to form (α 1 \rightarrow 3) linkages and synthesized a smaller and linear glucan polysaccharide with only (α 1 \rightarrow 6) linkages. Docking studies revealed that the introduction of the large aromatic amino acid residue tryptophan at position 940 partially blocked the binding groove, preventing the isomalto-oligosaccharide acceptor to bind in a favorable orientation for the formation of (α 1 \rightarrow 3) linkages. Our data showed that the reaction specificity of mutant glucansucrase GTF180 was shifted either to increased polysaccharide synthesis (L940A, L940S, L940E and L940F) or increased oligosaccharide synthesis (L940W). The L940W mutant is capable of producing a large amount of isomalto-oligosaccharides using released glucose from sucrose as acceptors. Thus, residue L940 in domain B is crucial for linkage and reaction specificity of GTF180. This study provides clear and novel insights into the structure-function relationships of glucansucrase enzymes.

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Truncation of domain V of the multidomain glucansucrase GTF180 of *Lactobacillus reuteri* 180 heavily impairs its polysaccharide-synthesizing ability

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Glucansucrases are exclusively found in lactic acid bacteria and synthesize a variety of α -glucans from sucrose. They are large multi-domain enzymes belonging to the CAZy family 70 of glycoside hydrolase enzymes (GH70). The crystal structure of the N-terminally truncated GTF180 of *Lactobacillus reuteri* 180 (GTF180- Δ N) reveals that the polypeptide chain follows a U shape course to form five domains, including domains A, B and C, which resemble those of family GH13 enzymes, and two extra and novel domains (domains IV and V), which are attached to the catalytic core¹. To elucidate the functional roles of domain V, we have deleted the domain V fragments from both the N- and C-terminal ends (GTF180- Δ N Δ V). Truncation of domain V of GTF180- Δ N yielded a catalytically fully active enzyme but with heavily impaired polysaccharide synthesis ability. Instead, GTF180- Δ N Δ V produced a large amount of oligosaccharides. Domain V is not involved in determining the linkage specificity and the size of polysaccharide produced as the GTF180- Δ N and GTF180- Δ N Δ V polysaccharides were identical in size and structure. The data indicates that GTF180- Δ N Δ V acts non-processively, frequently initiating synthesis of a new oligosaccharide from sucrose, instead of continuing the synthesis of a full size polysaccharide. Mutations L940E and L940F in GTF180- Δ N Δ V, which are involved in the acceptor substrate binding², restored polysaccharide synthesis almost to the level of GTF180- Δ N. These results demonstrated that interactions of growing glucan chains with both domain V and acceptor substrate binding sites are important for polysaccharide synthesis³.

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Identification and engineering of new family AA5 galactose oxidases

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Enzymatic oxidation can facilitate regiospecific modification of plant poly- and oligosaccharides for formation of aero- and hydrogels, as well as site-specific addition of new functional groups under mild aqueous conditions^{1,2,3,4}. Galactose oxidase (GaO) from *Fusarium spp.* is classified by the CAZy database as a family 5 auxiliary activity and has been used to oxidize several galactose-containing polysaccharides, thereby expanding their performance in bio-based materials. GaO oxidizes the C6-hydroxyl of terminal galactose substituents and is the only oxidase so far reported to oxidize the primary hydroxyl of terminal sugar-residues in polysaccharides.

Herein this poster, we describe C-terminal fusion of a family 3 carbohydrate binding module to the m-RQW variant of GaO (aka M₃)⁵, generating M₃-CBM3. While M₃ gained activity on glucose, the activity of M₃ on galactose decreased approximately 1000-fold⁵. Accordingly, in addition to constructing M₃-CBM3, three amino acid substitutions were herein introduced in an effort to retain widened substrate preference while maximizing catalytic efficiency. The activities of wild-type and engineered GaO were tested using 23 substrates, including galactose and glucose containing oligosaccharides and malto- and cellooligosaccharides. This analysis revealed that in addition to accepting a broader range of sugars for oxidation, M₃ is less sensitive to glycoside linkage type as compared to wild-type GaO. Although preliminary tests suggest that CBM3 fusion does not promote M₃ activity on cellulose or galactose containing polysaccharides bound to filter paper, M₃-CBM3 was 2.5 and 4 times more active on galactose and glucose than previously reported values for M₃ alone⁶. M₃-CBM3 was also able to functionally bind crystalline cellulose, promoting immobilization to cellulosic surfaces.

Acknowledgements

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Investigating synergism within multimodular glycoside hydrolases during wheat straw cell wall deconstruction

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In Nature, the plant-based organic carbon contained within plant cell walls is mainly recycled by the action of cellulolytic microorganisms, such as bacteria and fungi, which produce complex arrays of cell wall-degrading enzymes. Many of the enzymes that degrade plant cell wall polysaccharides are modular proteins, which contain single or multiple copies of both catalytic domain(s) and CBM(s). Cellulolytic microorganisms developed a couple of strategies to break down the complex plant cell wall; secrete free enzymes into their surroundings or produce large multi-component cell-bound structures, which harbour several enzymes displaying complementary activities. In both case, substrate is attacked by different enzymes acting together at different regions, and much more than sum of individual different enzymatic activities, synergism drives the efficiency of such system. Nevertheless, this complex process is still not completely understood. Despite an abundant literature about the role of CBM as an enhancer of the catalytic activity, we miss information about relationship between enzymes, CBMs and modularity *in planta*.

Here we report an original way to easily associate catalytic domains and CBMs to each other. Jo and In are small proteins (10.6 and 16.7 kDa respectively) from *Streptococcus pneumoniae*^{1,2}, which are able to spontaneously form one intradomain isopeptide bond. The xylanase Xyn11A from *Neocallimastix patriciarum* and the xylosidase Xyl43 from *Bacillus halodurans* were successfully expressed in *E.coli* and purified as Jo or In tagged proteins. In addition, cellulose specific CBM3a from *Clostridium thermocellum* and xylane specific CBM2b-1 from *Cellulomonas fimi* were also expressed and purified. Various combinations of these proteins were designed as free or solid support associated modular enzymes. Catalytic activities were investigated using chromogenic substrates, beach wood xylan and milled wheat straw.

The fine spatial proximity/synergism relationship in multimodular enzymes within the context of intact plant cell wall is presented in this work.

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Development of tailor-made ‘oxidative boosted’ enzyme mixtures for the bioconversion of targeted feed stocks

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The role of *Myceliophthora thermophila* and its enzyme aristocracy in the field of bioconversion of complex carbohydrates is well known today. Several cellulolytic enzymes have been discovered in this species which have been proven to have excellent efficiency on lignocellulosic substrates. With the latest introduction of the new enzyme group Lytic Polysaccharide Monoxygenases (LPMOs) and their proven ‘boosting’ effect on traditional cellulases, the dream of complete bioconversion of those recalcitrant polysaccharides is one more step closer to reality (1). However, the complexity of different feed stocks brings the need of customized enzyme proportions, specific for each of them, to attain maximum degradation without using excess enzyme. The lack of such customized enzyme cocktails results in either enzyme wastage or inefficient conversion of substrates, hence results in higher process cost. In this study, tailor-made enzymes mixtures targeted towards particular agricultural and forest feed stocks were tested for their ability to maximize hydrolysis yields. Various combinations of an LPMO (*MtLPMO9A*), along with a consortium of traditional cellulases and hemicellulases, all originated from *Myceliophthora Thermophila* and heterologously expressed in *Pichia pastoris* were used at various combinations (2,3). The synergistic effect of *MtLPMO* was optimized by varying the ratio of the various enzymatic components as well as the nature and amount of the reducing agent added in the degrading cocktail. Optimal combinations were predicted from suitable statistical models which were able to further increase hydrolysis yields, suggesting that tailor-made enzyme mixtures targeted towards a particular feed stock can help maximize hydrolysis yields. This work helps to lead the situation from ‘one cocktail for all’ to ‘tailor-made cocktails’ for targeted feed stocks, with better hydrolysis yields.

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Novel GH130 β -mannoside phosphorylases

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Glycoside hydrolase family (GH) 130 has been recently established and contains β -mannoside phosphorylases catalyzing the reversible phosphorolysis of β -mannosides to form α -D-mannose 1-phosphate with an inversion of its anomeric configuration. In this study, we characterized three GH130 homologues derived from *Bacteroides thetaiotaomicron* VPI-5482 (BT1033)¹ and *Thermoanaerobacter* sp. X-514 (Teth514_1788 and Teth514_1789)² to find novel phosphorylases showing unreported substrate specificity.

Acceptor specificity analysis of BT1033 in synthetic reaction showed that BT1033 utilized *N*-acetyl-D-glucosamine as the suitable acceptor. Furthermore, it was demonstrated that BT1033 catalyzed reversible phosphorolysis of β -1,4-D-mannosyl-*N*-acetyl-D-glucosamine in a typical sequential Bi Bi mechanism. We thus propose 4-*O*- β -D-mannopyranosyl-*N*-acetyl-D-glucosamine:phosphate α -D-mannosyltransferase as the systematic name and β -1,4-D-mannosyl-*N*-acetyl-D-glucosamine phosphorylase as the short name for BT1033. BT1033 gene is located in a gene cluster involved in complex-type N-glycan metabolism, suggesting that it plays a crucial role as intracellular phosphorolysis of β -1,4-D-mannosyl-*N*-acetyl-D-glucosamine liberated from the complex-type N-glycan by sequential glycoside hydrolase reactions.

Teth514_1788 and Teth514_1789 catalyzed the synthesis of β -1,2-oligomannan using β -1,2-mannobiose and D-mannose as the optimal acceptors, respectively. Kinetic analysis of phosphorolytic reaction toward β -1,2-oligomannan revealed that Teth514_1788 and Teth514_1789 prefer β -1,2-oligomannan containing a DP \geq 3 and β -1,2-mannobiose, respectively. These results indicate that these two enzymes are novel phosphorylases that exhibit distinct chain-length specificities toward β -1,2-oligomannan. Here, we propose β -1,2-oligomannan:phosphate α -D-mannosyltransferase as the systematic name and β -1,2-oligomannan phosphorylase as the short name for Teth514_1788 and β -1,2-mannobiose:phosphate α -D-mannosyltransferase as the systematic name and β -1,2-mannobiose phosphorylase as the short name for Teth514_1789. Based on the sequence analysis, we suggest that the two phosphorylases play a role in the intercellular phosphorolysis of β -1,2-oligomannan to supply α -D-mannose 1-phosphate for GDP-D-mannose biosynthesis in the bacterium.

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Discovery of 1,2- β -oligoglucan phosphorylase and large scale preparation of 1,2- β -glucan

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We characterized recombinant Lin1839 protein (Lin1839r) belonging to glycoside hydrolase family 94 from *Listeria innocua*. Lin1839r catalyzed the synthesis of a series of 1,2- β -oligoglucans (Sop_n; n denotes degree of polymerization) using sophorose (Sop₂) as the acceptor and α -D-glucose 1-phosphate as the donor. Kinetic analysis of the phosphorolytic reaction towards Sop₃ revealed that Lin1839r followed a sequential Bi Bi mechanism. The kinetic parameters of the phosphorolysis of Sop₄ and Sop₅ were similar to those of Sop₃, although the enzyme did not exhibit significant phosphorolytic activity on Sop₂. These results indicate that the Lin1839 protein is a novel inverting phosphorylase that catalyzes reversible phosphorolysis of 1,2- β -glucan with a degree of polymerization of ≥ 3 . We propose 1,2- β -oligoglucan: phosphate α -glucosyltransferase as the systematic name and 1,2- β -oligoglucan phosphorylase (OGP) as the short name for this Lin1839 protein.

We also produced 1,2- β -glucan enzymatically from 1.0 M sucrose and 0.5 M glucose by the combination of sucrose phosphorylase and OGP in the presence of 100 mM inorganic phosphate. Accumulation of 1,2- β -glucan in 2 L of the reaction mixture reached over 800 mM (glucose equivalent). Sucrose, glucose and fructose were removed after the reaction by yeast treatment. 1,2- β -Glucan was precipitated with ethanol to obtain 167 g of 1,2- β -glucan from 1 L of the reaction mixture.

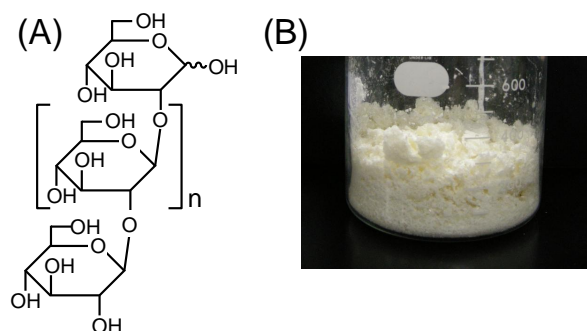


Fig 1. Structure of 1,2- β -glucan (A) and large-scale prepared 1,2- β -glucan (B).

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Exploring the secretomes of starch degrading fungi

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Starch is one of the most abundant renewable biopolymers in nature. Each year more than 2 billion tonnes of starch crops are harvested globally, making it an attractive resource for industrial applications [1,2]. Most applications of starch require the disruption of native granules or starch modification. Thus, developing efficient enzymes that can hydrolyse and modify native starch granules is of interest. The properties of starch granules, especially crystallinity, which varies between 15 to 45%, make complete enzymatic depolymerisation of starch challenging depending on botanic origin and processing [3].

In contrast to lignocellulose matrices [4], much less effort has been imparted on discerning the enzyme cocktails deployed by fungi, both with respect to class, composition and proportion, for the degradation of starch. This insight is likely to have impact on the efficiency of industrial starch processing.

Fungi are renowned for producing an arsenal of carbohydrate active enzymes (CAZymes) [5] to harvest energy from various glycans. The main focus of the present study is to examine the degradation machinery of starch by filamentous *Aspergillus* spp., which exhibit tremendous ecological, biological and metabolic diversity and are used industrially for recombinant enzyme production.

Here we present an *in silico* analyses of the fungal CAZymes plausibly involved in the degradation of starch by fungi. The growth of selected *Aspergillus* strains on starchy substrates of different properties will be assessed and proteomics tools will be used to analyse complex mixture of enzymes that are secreted (i.e. the secretomes) to mediate growth on starch. The latest results from this study will be presented and discussed.

Acknowledgements

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Chemo-enzymatic synthesis of chitoheptaose using a glycosynthase derived from an inverting chitinase with an extended binding cleft

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We successfully created the first glycosynthase derived from a “loopless” family GH19 chitinase from moss (*BcChi-A*), whose oligosaccharide-binding cleft consists of only four subsites -2, -1, +1, and +2 (Ohnuma *et al.*, *Biochem. J.*, 444, 437-443, 2012). However, the chain-lengths of the synthetic products were limited, because the “loopless” enzyme could accept only oligosaccharides with shorter chains as a donor or an acceptor substrate. Here, we report a glycosynthase derived from a “loopful” GH19 chitinase from rye seeds (*RSC-c*) whose binding cleft consists of subsites -4, -3, -2, -1, +1, +2, +3, and +4. When the wild-type *RSC-c* was incubated with α -(GlcNAc)₃-F [α -(GlcNAc)₃ fluoride], (GlcNAc)₃ and hydrogen fluoride were produced through the Hehre resynthesis–hydrolysis mechanism. Then, mutations were introduced into Glu89 acting as a catalytic base and Ser120 holding a nucleophilic water molecule, to produce two single mutants, E89G and S120A. E89G synthesized a small amount of (GlcNAc)₇ from α -(GlcNAc)₃-F in the presence of (GlcNAc)₄, while S120A with the highest F⁻-releasing activity successfully produced a large amount of (GlcNAc)₇. However, a fraction of (GlcNAc)₇ synthesized by S120A was decomposed by its own residual hydrolytic activity. Thus, we finally produced a double mutant E89G/S120A, which produced a large amount of (GlcNAc)₇ without decomposition. The double mutation completely abolished the hydrolytic activity and maintained moderate F⁻-releasing activity. E89G/S120A, which was the best glycosynthase, can produce chitin oligosaccharides with longer chains.

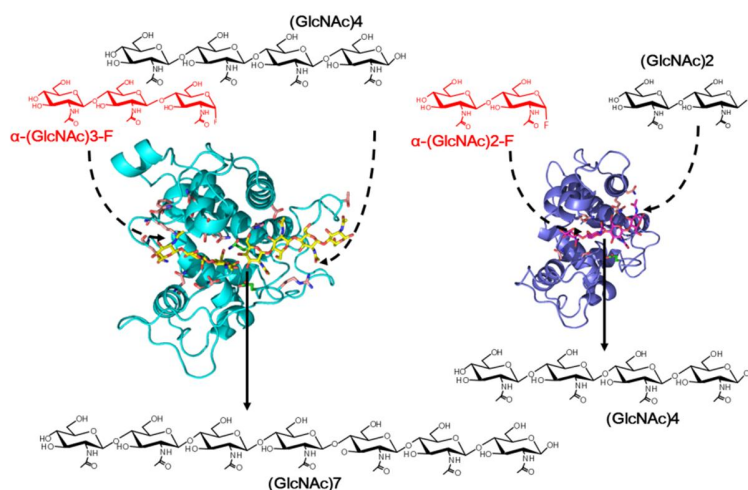


Fig 1. Chemo-enzymatic synthesis of chitin oligosaccharide using a glycosynthase derived from an inverting GH19 chitinase

A transglycosylation of catalytic nucleophile mutant of GH97 α -galactosidase with an external nucleophile

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α -Galactoside is one of the most important classes of carbohydrates, including such as α -gal epitope [α -Gal-(1 \rightarrow 3)- β -Gal-(1 \rightarrow 4)-GlcNAc-R or α -Gal-(1 \rightarrow 3)- β -Gal-(1 \rightarrow 3)-GlcNAc-R], globosyl-Gb2 [α -Gal-(1 \rightarrow 4)- β -Gal-R], -Gb3 [α -Gal-(1 \rightarrow 4)- β -Gal-(1 \rightarrow 4)- β -Glc-R], and raffinose [α -Gal-(1 \rightarrow 6)- α -Glc-(1 \leftrightarrow 2) β -Fru]. The α -gal epitope and Gb3/Gb2 are of major clinical significance and raffinose receives a lot of attention as a prebiotics.

Most, if not all, of α -galactosides are synthesized by NDP-forming glycosyltransferases, but an α -galactosidase also catalyzes the formation of α -galactoside in a transglycosylation reaction. To ensure the maximum yield of the transglycosylation product is the biggest challenge, because the normal hydrolysis reaction would reduce the transfer product. Glycosynthase, having a site-directed mutation at a nucleophile catalyst of a retaining glycosidase, is capable of catalyzing glycosyl transfer from wrong glycosyl fluoride to acceptor alcohols without hydrolysis of the product, and can overcome this problem. However, the glycosyl donor substrate of the glycosynthase formed from the α -galactosidase is less stable β -galactosyl fluoride, and it is expected to make a low yield of the transglycosylation product. We thus exploit somewhat more stable α -galactosyl fluoride (α -GalF) as the glycosyl donor substrate of the catalytic nucleophile mutant of glycoside hydrolase family 97 α -galactosidase, *BtGH97b*, with an external nucleophile, to establish the maximum yield of the product.

The glycosyl transfer from α -GalF to lactose by the catalytic nucleophile mutant of *BtGH97b*, D415G, occurs with a formate ion as the nucleophile. NMR-analysis indicates that the product is β -lactosyl α -galactopyranoside [α -D-Galp-(1 \leftrightarrow 1) β -D-Glcp-(4 \leftarrow 1) β -D-Galp], which is a unique non-reducing sugar. Sodium azide can more effectively restore the catalytic reaction, but no transglycosylation product is detected and an accumulation of β -galactosyl azide is observed. A kinetic analysis suggests that the less reactivity of β -galactosyl azide results in the accumulation. Glucose, xylose, maltose, and cellobiose can also act as acceptor, with the yields of 75% (glucose), 90% (xylose), 90% (maltose), and 75% (cellobiose), respectively, but galactose cannot act as acceptor.

A crystal structure of D415G in complex with β -lactosyl α -D-galactoside allows to investigate the molecular aspect of substrate binding site of *BtGH97b*. Two aromatic residues at the entrance of the active-site pocket of *BtGH97b* make the acceptor binding site narrow, and this structural feature can be responsible for the formation of α -Gal-(1 \leftrightarrow 1)- β -Glc linkage and for inability to form the α -galactosyl galactose linkage. The narrow entrance might prohibit the galactose residue having axial OH-4 from binding to the acceptor site.

The abstract has been withdrawn

Design of a nano-system targeting the tumor micro-environment for the treatment of tumor by inhibition of a specific β -endoglycosidase responsible for angiogenesis

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Most of the available anticancer agents cannot distinguish between cancerous and healthy cells, leading to systemic toxicity and undesired side effects. The development of nanoscale tumor-targeted delivery systems may resolve these problems. One of the most common strategy involves coupling a nanocarrier with a specific ligand. This ligand should be sensitive to biological and environmental signals such as pH, temperature, and specific enzymes. Highly-interesting are enzyme sensitive systems exploiting over-expressed disease-associated enzymes to trigger drug release or fluorescence. In case of cancer, these tools are perfectly adapted as the tumor micro-environment shows chemical and structural particularities including overexpressed extracellular protease and glycosidase.

The main strategy used in this project is the production of an inactive glycopeptidic precursor that will be activated when cleaved by a protease hyper-secreted and activated in tumoral microenvironment: the Cathepsin-D¹. Then, the released glycosidic part is designed to inhibit an endoglycosidase responsible for the angiogenesis² phenomenon: the Heparanase.

We are also working on adding a FRET fluorescence system on the peptidic part that will show fluorescence as the cleavage occurs.

The purpose of this project is thus to prepare by a standard procedure, a functional nano-tool to be used for both measurement of cellular activities of biomarkers involved in cancers and the release of a drug.

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Diversity of xylan deacetylases of family CE16: action on acetylated aldotetrauronic acid and glucuronoxylan

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Studies of enzymatic degradation of acetylated hardwood glucuronoxylan showed that microbial acetylxyylan esterases are capable of liberating acetic acid from singly and doubly acetylated xylopyranosyl (Xylp) residues, but not from position 3 of Xylp residues α -1,2-substituted with 4-*O*-methyl-D-glucuronic acid (MeGlcA) [1-3]. This acetyl group was found to be attacked by family CE16 deacetylases [4,5], but only in aldotetrauronic acid, MeGlcA³Xyl₃, acetylated at the non-reducing end Xylp residue, which is a product of GH10 xylanase degradation of the polysaccharide [6-8]. The monoacetylated aldotetrauronic acid was isolated from an enzymatic aspen acetylglucuronoxylan hydrolysate by a combined action of GH10 xylanase, CE6 acetylxyylan esterase and GH67 α -glucuronidase. The monoacetylated MeGlcA³Xyl₃ did not serve as substrate for the GH67 α -glucuronidase. Here we report that in the monoacetylated aldotetrauronic acid the 3-*O*-acetyl group migrates to position 4 which was originally involved in the glycosidic linkage. However, the migration is much slower than in linear xylooligosaccharides acetylated at the non-reducing end Xylp residue [9]. The MeGlcA side residue at position 2 obviously rigidifies the xylopyranosyl ring and hampers the acetyl group migration. Examination of the mode of action of *Trichoderma reesei* CE16 acetylsterase by ¹H-NMR showed that the enzyme liberates acetic acid from the aldotetrauronic acid from both position 3 and 4, and at about the same rate. Surprisingly, another CE16 deacetylase from *Podospira anserina* behaved differently. In the monoacetylated aldotetrauronic acid the *P. anserina* enzyme deacetylated exclusively position 4 although the enzyme deacetylated all positions 2, 3 and 4 on the non-reducing end of methyl β -D-xylotrioside. In contrast to *T. reesei* CE16, the *P. anserina* enzyme also deacetylated all singly and doubly acetylated Xylp residues in the polymeric substrate. These results outline a strategy for complete enzymatic degradation of hardwood acetylglucuronoxylan isolated by non-alkaline extraction procedures. At the same time, they illustrate variation among CE16 deacetylases, emphasizing the need to characterize more members of this interesting enzyme family.

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Conformational studies on trivalent acetylated mannoside clusters

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The immunostimulatory properties of β -(1 \rightarrow 2)-linked mannosides of natural origin have been studied for some time already¹ and considerable amount of effort has gone into the synthesis² and biological evaluation^{2,3} of such compounds. One of the underlying reasons for the observed biological activity is believed to be the unique helical conformation resulting from the rare β -(1 \rightarrow 2)-linkage, which is apparent even in molecules as small as tri- and tetrasaccharides. We have previously screened a large number of fully synthetic mannose-based structures for potential use as adjuvants in allergen immunotherapy.⁴ The screened molecules have ranged from simple mono- and oligosaccharides to oligovalent clusters containing also acetylated compounds. Among these molecules, the trivalent cluster **1** was found to be a potent candidate for further investigations showing promising activity both *in vitro* and *in vivo*.^{4c,5} Similar *in vitro* activity has recently been demonstrated also for the closely analogous structure **2** with the linker elongated by one $-\text{CH}_2-$ moiety.

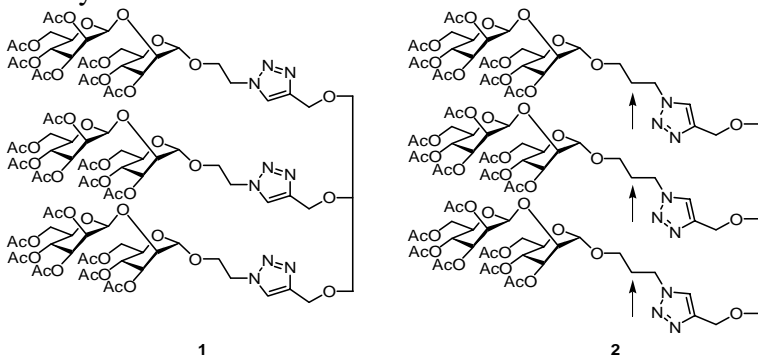


Fig 1. Trivalent acetylated mannosides studied in this work.

In the present work, these two molecules were subjected to comprehensive NMR studies and molecular dynamics simulations in order to elucidate their behavior in solution. This provides valuable information about how such compounds may fit into the binding pockets of receptors on cell surfaces which, in turn, is important for understanding their biological activities.

Acknowledgements Financial support from the Academy of Finland and the COST Action CM1102 MultiGlycoNano is gratefully acknowledged.

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The conformational free-energy landscape of β -xylose reveals a two-fold catalytic itinerary for β -xylanases

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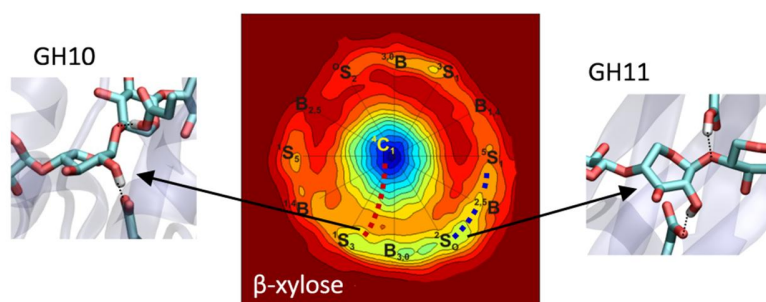
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Unraveling the conformational catalytic itineraries¹ of glycoside hydrolases (GHs) is a growing topic of interest in glycobiology, with major impact in the design of GH inhibitors. β -xylanases are responsible for the hydrolysis of glycosidic bonds in β -xylans, a group of hemicelluloses of high biotechnological interest that are found in plant cell walls. The precise conformations followed by the substrate during catalysis in β -xylanases have not been unambiguously resolved, with three different pathways being predicted from structural analyses. In this work, we compute the conformational free energy landscape (FEL) of β -xylose to predict the most likely catalytic itineraries followed by β -xylanases. The calculations are performed by means of *ab initio* metadynamics, using the Cremer-Pople puckering coordinates as collective variables.² The computed FEL supports only two of the previously proposed itineraries, ${}^2S_0 \rightarrow [{}^{2.5}B]^\ddagger \rightarrow {}^5S_1$ and ${}^1S_3 \rightarrow [{}^4H_3]^\ddagger \rightarrow {}^4C_1$, which clearly appear in low energy regions of the FEL. Consistently, 2S_0 and 1S_3 are conformations preactivated for catalysis in terms of free energy/anomeric charge and bond distances. The results⁴ however exclude the ${}^0E \rightarrow [{}^0S_2]^\ddagger \rightarrow B_{2,5}$ itinerary that has been recently proposed for a family 11 xylanase.⁴ Classical and *ab initio* QM/MM molecular dynamics simulations reveal that, in this case, the observed 0E conformation has been enforced by enzyme mutation. These results add a word of caution on using modified enzymes to inform on catalytic conformational itineraries of glycoside hydrolases.



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Structural and biochemical characterization of endo-acting chondroitin AC lyase a family 8 polysaccharide lyase (*PsPL8a*) from *Pedobacter saltans* DSM 12145

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Chondroitin sulphate (CS) is a sulphated glycosaminoglycan (GAG) composed of an unbranched chain of alternating sugars N-acetylgalactosamine and glucuronic acid. CS is the major polymeric component of animal extracellular matrix, acting as cell surface receptor. It is involved in cell signalling, cell proliferation, tumor growth and metastasis (1). The gene encoding *PsPL8a* (2034 bp) from *Pedobacter saltans* belonging to family 8 polysaccharide lyase was cloned, expressed and purified as soluble protein of molecular size ~77 KDa. Purified *PsPL8a* showed specificity towards both chondroitin 4 sulphate (C4S) and chondroitin 6 sulphate (C6S), showing it to be chondroitin AC lyase. *PsPL8a* was found to be endo-acting as determined by Thin Layer Chromatography and Mass Spectrometry analysis of degraded products of C4S. Protein melting studies by UV-Visible spectroscopy showed that protein starts melting at 45°C and completely melts at 60°C. Effect of chaotropic agents like urea and guanidine hydrochloride on structural integrity of *PsPL8a* was studied by fluorescence spectroscopy and a red shift was observed at 5 M Urea and 3 M GdHCl indicating loss of structural integrity. 3D structure of *PsPL8a* was developed by homology modeling using Modeller v9.14. *PsPL8a* sequence was aligned with best matched template of known chondroitin AC lyase with pdb id 1HM2 from *Flavobacterium heparinus* of PL8 family. Modeled structure was refined and energy minimization was carried out at 3D refine protein structure refinement server. The modeled structure of enzyme showed N-terminal domain containing predominantly α -helices forming (α/α)₆ toroidal topology, while C-terminal domain contains predominantly β strands arranged in 4 layered β -sheet sandwich. The resulting structure was validated using the tools available on the SAVES and ProSA-web server. Ramachandran plot revealed that 98.5% of the residues lie in the allowed regions, 1.2% lies in the generously allowed regions while only 0.5% of the residues lie in the disallowed regions. Secondary structure elements were predicted by PSIPRED server which showed presence of α helix (24.92%), β strands (21.68%) and rest, the coils. Far UV Circular Dichroism spectrum of *PsPL8a*, showed presence α helix (27.31%), β strand (22.7%) and rest were the coils corroborating the predicted data. *PsPL8a* is the first chondroitin AC lyase reported from *Pedobacter saltans*.

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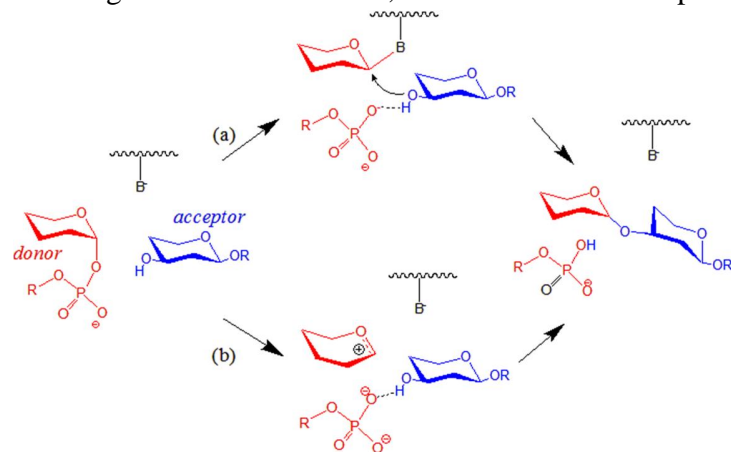
Molecular mechanisms of retaining glycosyltransferases. Insight from QM/MM metadynamics simulations

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The catalytic mechanism of nucleotide-sugar dependent glycosyltransferases (GTs), especially those that act with retention of anomeric configuration, remains one of the intriguing unanswered questions in glycobiology. In contrast to the well-characterized mechanistic strategies used by glycoside hydrolases (GHs) to catalyze the cleavage of glycosidic bonds, the mechanisms of retaining GTs remain unclear, with both double displacement (a) and front-face (b) mechanisms



being proposed. By means of QM/MM metadynamics simulations, based on Density Functional Theory (DFT), we initially demonstrated that the front-face mechanism is feasible for trehalose-6-phosphate synthase (OtsA) a GT lacking a putative nucleophile residue on the beta face of the donor sugar, thanks to the formation of a short-lived oxocarbenium-ion-like species.¹ Similar results have been obtained for polypeptide GalNAc-transferase 2 (GalNAc-T2),³ one of the key enzyme

isoforms controlling human protein O-glycosylation. In contrast, a GT with a putative nucleophile residue, such as α 3-galactosyltransferase (α 3GalT), was found to operate via a double-displacement mechanism, with the formation of a glycosyl-enzyme covalent intermediate.² A detailed picture of the atomic rearrangement during the complete reaction pathway will be provided and differences between the three enzymes will be analyzed.

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Comparative analysis of transcriptomes and secretomes of the white-rot fungus *Dichomitus squalens* cultured in lignocellulosic substrates

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White-rot fungi are essential organisms in the degradation of plant biomass and thus play an important role in the global carbon cycle. In addition to their ability to mineralize the aromatic lignin polymer, white-rot fungi produce enzymes that degrade plant cell wall polysaccharides into oligosaccharides and monomeric sugars that they can use as carbon source. These enzymes are important tools for biotechnology, as the products of their catalysis can be used as precursors of bio-based fuels and chemicals.

Dichomitus squalens is a white-rot basidiomycete capable of efficient cellulose and lignin degradation on softwood. The focus of this work was to dissect the enzyme combinations used by the *D. squalens* in the degradation of different types of plant-derived biomass. For that, *D. squalens* was grown on hardwood, softwood, and residues from monocots and dicots as carbon sources and sampled at two time points for transcriptome and secretome analyses. Extracellular plant biomass degrading enzyme activity profiles were also determined during the growth of *D. squalens* on these plant biomass materials. These analyses provide an in-depth appreciation of the mechanisms used by *D. squalens* in the degradation of diverse plant-derived biomass. Further, these results are also expected to enhance the discovery and use of novel enzymes in biotechnological applications.

Acknowledgements

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Biochemical characterization and crystal structure of a novel GH127 β -L-arabinofuranosidase

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Arabinan is a branched polysaccharide and is part of pectin, one of the components in the plant cell wall. The arabinan backbone consists of α -1,5-linked L-arabinofuranosyl units, and is decorated mainly with α -1,2- and α -1,3-linked arabinofuranosides. Unlike the widespread α configuration of arabinofuranosyl, β -arabinofuranosyl residues are relatively rare in native polysaccharides.

Geobacillus stearothermophilus T-6 is a thermophilic soil bacterium that possesses an extensive system for the utilization of L-arabinan. The system is composed of five transcriptional units, one of which contains eleven genes (*abnEFJ-abnA-abfBA-araJKLMN*). This operon encodes for an arabino-oligosaccharides transporter system, two α -L-arabinofuranosidases (Abf51A and Abf51B), and several genes encoding for an alternative arabinose utilization pathway. The last gene in the operon, *ara127N*, encodes for a β -L-arabinofuranosidase and shows activity towards both synthetic and natural substrates containing β -L-arabinofuranoside residues. The k_{cat} value of Ara127N towards pNP- β -L-arabinofuranoside in pH 6.5 is 1.5 sec^{-1} , K_m is 0.57 mM and the calculated value of k_{cat}/K_m is $2.6 \text{ sec}^{-1} \cdot \text{mM}^{-1}$. The crystal structure of Ara127N has recently been determined (at 2.30 Å resolution) and it appears to have a metal-binding motif with Zn^{2+} coordinated by glutamate and cysteines in the active site. Inductive coupled plasma (ICP) measurements show that Ara127N contains 0.8 mol of zinc per mol of enzyme. Amino acid sequence analysis revealed three conserved cysteine residues, Cys329, Cys406 and Cys407 that could be involved in metal binding. Preliminary results demonstrated that a single mutation of Cys406Ala abolished metal binding and affected the catalytic activity, suggesting the involvement of the metal in the catalytic mechanism.

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Xylooligosaccharides (XOs) from xylan extracted from quinoa (*Chenopodium quinoa*) stalks

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Xylooligosaccharides (XOs) were obtained from xylan, extracted from alkaline pretreated, milled Quinoa stalks through the action of thermostable xylanases. Alkaline pre-treatment with NaOH (2%) resulted in a total yield of 10.853 g of xylan/100 g raw-material, of which 62% and 38 % was obtained between soluble and insoluble fractions respectively. Monosaccharides analysis of the extracted xylan showed the major components to be xylose:arabinose in a molar ratio of 12.3:1. Six different xylanases variants including Xyn10AK80R, HXyn10A, Xyn10AH from *Bacillus halodurans*, Xyn10AF, Xyn10ACat from *Rhodotermus marinus* and PENTOPAN[®] were evaluated for their ability to transform the soluble/insoluble xylan in water or buffer to XOs with degree of polymerization (DP) in the range 2 – 6. The major XOs obtained with 5 of the 6 thermostable xylanases were xylobiose (X2) and xylotriose (X3). The major XOs product using Xyn10AK80R and Xyn10AH was X2, while treatment with Xyn10AF, Xyn10ACat and PENTOPAN[®] resulted X3 from both soluble and insoluble xylan fractions. XOs with DP 4 to 6 were only produced in low amounts, using all enzymes. The enzyme variant HXyn10A produced all XOs, but in low or negligible concentrations. Although XOs production from both soluble and insoluble xylan followed the same trend, there was a significant difference ($p < 0.05$) between the concentration of XOs obtained in the respective xylan fraction, with higher yields of X2 and X3 using soluble xylan. In contrast, reactions run in buffer or water did not show a significant difference ($p > 0.05$), showing that either water or buffer reaction media are good for these enzymes. In conclusion, XOs were successfully obtained using thermostable xylanases acting on xylan extracted from Quinoa stalks; a novel agricultural waste which could be a useful material for production of prebiotic xylooligosaccharides.

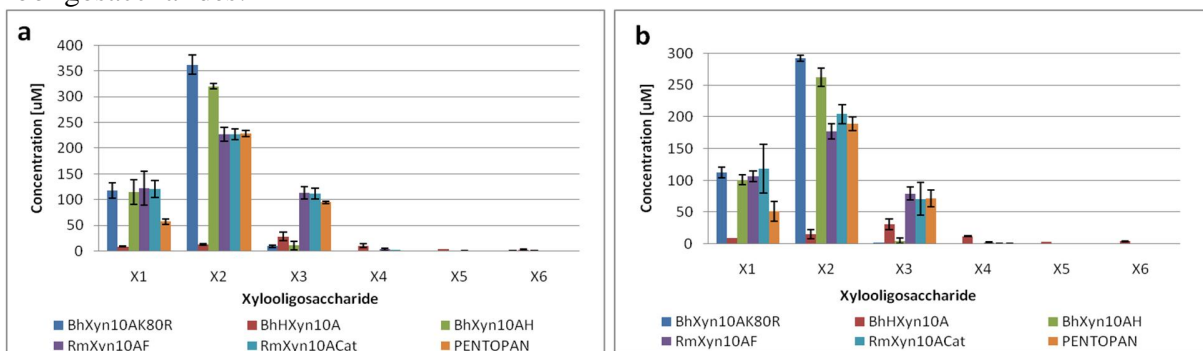


Fig 1. Xylose (X1), xylobiose (X2), xylotriose (X3), xyloetraose (X4), xylopentaose (X5) and xylohexaose (X6) obtained after reaction with thermostable xylanases on soluble (a) and insoluble (b) xylan. *B. halodurans*: Xyn10AK80R, HXyn10A, Xyn10AH; *R. marinus*: Xyn10AF, Xyn10ACat and PENTOPAN[®].

Acknowledgements

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Conformational study on homoallylic polyol derived from D-mannose

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Terminally unsaturated and diastereomerically pure polyol derived from D-mannose shows spontaneous aggregation behavior in water solution. In order to clarify this unforeseen phenomenon, conformational study based on NMR spectroscopic studies was pursued. Ab initio geometry optimizations using the COSMO-solvation model were used to quantify the conformational effects observed in the NMR analysis. The recently performed X-ray diffraction studies provided new insights into the conformation and aggregation behavior. In this poster, we will describe our twisting path with polyol **1** from visual observation in the laboratory towards detailed conformational analysis.

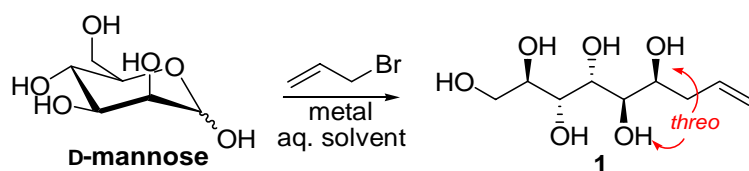


Fig 1. Synthesis of diastereomerically pure polyol **1** from D-mannose

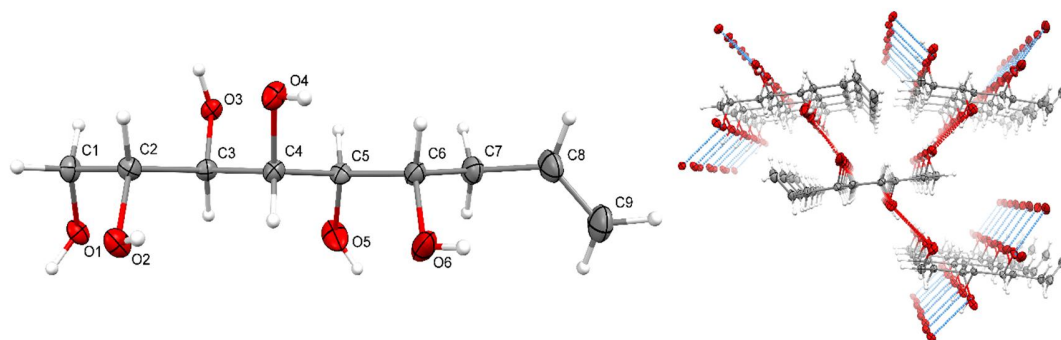


Fig 2. Left: asymmetric unit of crystal structure of **1**. Right: intermolecular hydrogen bonding pattern (dotted lines) observed in crystal structure of **1** and viewed along crystallographic *c*-axis.

Protein stability engineering by structure-guided chimeragenesis

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β -glucosidases are key enzymes during conversion of plant biomass glucans to the major final degradation product D-glucose. We have identified a GH family 3 β -glucosidase from *Fusarium verticilloides* (Fv3C) that displays good performance on a panel of biomass substrates. However, when this enzyme was expressed in *Trichoderma reesei*, this molecule was proteolytically clipped. MS analysis, N-terminal sequencing, and X-ray crystallography characterizations showed that the protein got clipped within the C-terminal third domain of the molecule resulting in a significant loss of activity performance. Attempts to fix the integrity of the Fv3C molecule locally, including site-specific mutations, loop replacement or deletions, linker or disulfide bridge insertions, and removal or introduction of N-linked glycosylation sites, were unsuccessful. Alternatively, we applied a “global” approach via interchanging regions between Fv3C and homologs. Replacing the C-terminal domain of Fv3C repaired the clip site, but surprisingly created another one in a loop upstream of the previous clip site. This loop is specific only to *Fusarium* GH3 β -glucosidases, and shortening this sequence to a single Gly residue, or to a corresponding region from a homologous GH3 enzyme led to new and improved molecules that are produced in intact form by *T. reesei*. The three-molecule hybrid performed well and remained stable throughout extended saccharification assays.

Evaluation of microbial production of exopolysaccharide by *Rhodothermus marinus* strains: potential for industrial biotechnology

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The formation of extracellular polysaccharides (EPS) by *Rhodothermus marinus* DSM 4252 and *Rhodothermus marinus* 493, two wild type species of thermophilic bacteria has been screened in different culture media. Marine broth containing either 1 or 10 g/l of glucose, sucrose, lactose, and maltose, separately was used. The results showed that these two strains have the ability to produce and release the EPS. Marine broth containing 10 g/l lactose showed the highest production of EPS in both strains. Besides, EPS production was mainly during the stationary phase. The monosaccharide composition of the produced exopolysaccharides was analyzed and quantified. The results suggested a heteropolymer structure for produced EPS of both strains. The most abundant monomers were xylose, arabinose, and mannose in all media in both strains. Also, the presence of glucose, galactose, and raffinose was varied depending on the type of media used for production of EPS. The EPS of *R. marinus* DSM 4252 included high quantity of xylose, while in *R. marinus* 493 arabinose had the highest amount compared to the other produced monosaccharides. The molecular mass of produced EPS by these two strains was determined by size exclusion chromatography technique using Sephacryl S-200 and Sephacryl S-500 columns. These results lead us for further studies aimed at increasing the interest in the application of produced EPS of *Rhodothermus marinus* in food, pharmaceutical, and wastewater treatment industries by optimizing the condition for high production of EPS and characterizing its physicochemical properties.

Acknowledgements

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Characterization of two trisaccharides produced in the presence of lactose by *Weissella confusa* dextranucrase

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Many lactic acid bacteria are able to synthesize polymeric glucans or fructans from sucrose. Dextran-producing *Weissella* species have received recently increasing attention due to their potential in food fermentations [1], especially in sourdough production. However, in the presence of suitable acceptors, such as disaccharides, oligosaccharides are produced in addition to elongating polysaccharide. These acceptor reactions of *Weissella* dextranucrases have not been investigated except for maltose, which is the best known acceptor. Lactose is naturally present in milk. Thus it is of interest to identify the acceptor products which may be formed during fermentation of milk-based foods by *Weissella* strains.

The main oligosaccharides produced by the recombinant *W. confusa* VTT E-90392 dextranucrase [2] in the presence of lactose and sucrose were studied in this work. Beside the main product, interestingly another oligosaccharide was observed in the HPAEC-PAD chromatogram. The two products were isolated by gel filtration and subject to detailed characterization. They were both trisaccharides, and their structures were elucidated by NMR spectroscopy as 2^{Glc}- α -Glc_p-lactose and isomelezitose (6^{Fru}- α -Glc_p-sucrose), respectively. The products were also analyzed by ESI-MS/MS. The further use of labeled ¹³C₆^{Glc} sucrose in the reaction enabled the detailed identification of fragmentation pattern of these two trisaccharides in the positive and negative ion MSⁿ analysis.

2^{Glc}- α -Glc_p-lactose has earlier been identified as the lactose acceptor product of *L. mesenteroides* B-512F dextranucrase, and has shown potential as prebiotics. However, the dextranucrase-catalyzed synthesis of isomelezitose has not been reported before. The fragmentation pathways of the two trisaccharides in MSⁿ are studied for the first time. The information obtained will be useful for future LC-MS/MS characterization of oligosaccharides of similar structure.

Acknowledgements This study was supported by the Academy of Finland (MT) via WISEDextran project, the ABS Graduate School (QS) and the Raisio plc Research Foundation (MJ) in Finland.

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α -L-Fucosidase from *Fusarium proliferatum* LE1: specificity and transglycosylation abilities

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Oligosaccharidic chains of many L-fucose-containing glycoconjugates involved in many vital biochemical events. Fucosylated glycans play important roles in various physiological and pathological processes [1]. One possible approach to study the role of fucosylation is the release of L-fucose by specific enzyme such as α -L-fucosidase. Additionally, the ability of some of these enzymes to catalyze the transfucosylation reactions indicates the potential to use them in biotechnology.

Recently, we have discovered and identified a new strain of filamentous fungus *Fusarium proliferatum* LE1 (deposition number in Russian Collection of Agricultural Microorganisms RCAM02409), which is a producer of α -L-fucosidase [2]. This strain appeared to highly produce α -L-fucosidase when grown in a medium containing L-fucose, which is the reaction product of the enzyme. As a result of four chromatographic stages, we obtained a purified enzyme with a molecular mass of about 60 kDa. The α -L-fucosidase exhibits predominant selectivity towards α 1 \rightarrow 4-*O*-fucosyl bonds in difucosaccharides with different types of linkages; hydrolyzes *p*-nitrophenyl α -L-fucopyranoside (*p*NPFuc) with $K_m=1.1 \pm 0.05$ mM and $k_{cat} = 35.7 \pm 1.8$ c⁻¹. L-fucose was found to be a competitive inhibitor of hydrolysis of *p*NPFuc with the inhibition constant $K_I = 0.2 \pm 0.05$ mM. As a result of NMR analysis of the stereochemical course of the enzyme-catalyzed *p*NPFuc hydrolysis, we observed retention mechanism of the α -L-fucosidase action. α -L-Fucosidase from *F. proliferatum* LE1 was able to catalyze fucosylation of aliphatic alcohols but no transglycosylation products were found in the reaction with *p*NPFuc as a donor and an acceptor. Analysis of sequenced gene encoding the synthesis of α -L-fucosidase from *F. proliferatum* LE1 revealed classification of the enzyme to the glycosyl hydrolase family 29 (The Carbohydrate-Active Enzymes database (CAZy; <http://www.cazy.org>) [3].

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Hemicellulases in total hydrolysis of wood-based substrates

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Woody raw materials are greatly potential renewable sources for fuels and chemicals, which can be produced using various chemical and biochemical routes. In recent years there has been increasing global interest to use these feedstocks as raw materials for microbial fermentations in bioproduction. This so called sugar route is based on efficient hydrolysis of natural biopolymers to sugars which is in most cases carried out by enzymes. Developing efficient enzyme mixtures requires knowledge of the limiting and enhancing factors in the action of hydrolytic enzymes. In this work we studied the role of hemicellulose-hydrolyzing enzymes in the mixtures, and simultaneously gained information of the role of hemicellulose when using woody raw materials as source of monosaccharides for sugar route.

Various woody raw materials (spruce chemical pulp, spruce pretreated either by steam explosion or catalytic oxidation, nanofibrillated cellulose and crystalline softwood cellulose) were studied in hydrolysis using designed enzyme mixtures. The enzymes used were purified preparations from fungi *Trichoderma reesei* (TrCel5A, TrCel6A, TrCel7A, TrCel7B, TrMan5A, TrXyn11) or *Aspergillus niger* (a GH3 β -glucosidase). The effect of the mixture composition on the release of carbohydrates from the substrates was analyzed. The adsorption of the single components of the mixture on several substrates was also analyzed and will be discussed in relation to hydrolysis efficiency.

The results showed the great importance of hemicellulolytic enzymes in total hydrolysis of wood-based cellulosic substrates. On the other hand, the results also indicated that a part of hemicellulose is located deeply embedded in the cellulosic matrix, and evidently prevents efficient enzymatic saccharification of wood-based cellulose, unless processed by additional corresponding enzymes. Both cellulose and lignin fraction of biomass seemed to adsorb hemicellulases during hydrolysis.

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Structure-function relationships in the active site of the blue mussel β -mannanase *MeMan5A*

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Hemicelluloses are important components of lignocellulosic biomass, and their enzymatic degradation has great promise for substituting fossil-based products with renewable bio-based alternatives. A variety of enzymes are required to efficiently degrade these hemicelluloses. One such enzyme type is β -mannanases, which hydrolyze the backbone of β -1,4-mannans. Some β -mannanases are also able to synthesize new mannosidic bonds or transfer other molecules onto manno oligosaccharides, through a process called transglycosylation. In addition to their innovative potential, studying β -mannanases is also important from a biological perspective, since many organisms use β -mannanases in order to utilize mannans as a source of nutrients. The blue mussel *Mytilus edulis* is one such organism, having a β -mannanase (*MeMan5A*) in its digestive tract. Previous work [1] suggests that *MeMan5A* is highly efficient in degrading mannan-based substrates, and that it may also be able to perform transglycosylation. The +2 sugar-binding subsite seems to be particularly important for these properties. In the current work, two tryptophans in the +2 subsite of *MeMan5A* were substituted with alanines, leading to a reduction in both hydrolytic efficiency and transglycosylation capacity, as well as affecting substrate binding in the active site. This is consistent with studies of other glycoside hydrolases, where aglycone (+) subsites were also found to be important for hydrolysis and transglycosylation [2, 3]. The effect of substituting both tryptophans was greater than either residue by itself, possibly suggesting some degree of cooperativity between these two residues in the +2 subsite of *MeMan5A*. Future work intends to study the structural basis of the observed effects using nuclear magnetic resonance (NMR). Such studies of structure-function relationships in β -mannanases could enable fine-tuning enzyme properties to create the most efficient β -mannanase for any given application.

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Engineering a thermostable fungal GH10 xylanase, importance of N-terminal amino acids

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Xylanases are used in many industrial processes including pulp bleaching, baking, detergent and the hydrolysis of plant cell wall in biofuels production. In this work we have evolved a single domain GH10 xylanase, Xyn10A_ASPNG, from *Aspergillus niger* to improve its thermostability. We introduced a rational approach involving as the first step a computational analysis to guide the design of a random mutagenesis library in targeted regions which identified thermal important residues that were subsequently randomly mutagenized through rounds of iterative saturation mutagenesis (ISM). Focused on five mutational positions, four rounds of ISM have generated a quintuple mutant 4S1 (R25W/V29A/I31L/L43F/T58I) which exhibited thermal inactivation half-life ($t_{1/2}$) at 60°C that was prolonged by 30 folds in comparison with wild-type enzyme. Furthermore, the mutant melting temperature (T_m) increased by 17.4°C compared to the wild type. Each of the five mutations in 4S1 was found to contribute to thermoresistance, but the dramatic improvement of enzyme thermoresistance of 4S1 was attributed to the synergistic effects of the five mutations. Comparison of biochemical data and model structure between 4S1 and the wild-type enzyme suggested that the N-terminal coil of the enzyme is important in stabilizing GH10 xylanases structure. Based on model structure analyses, we propose that enforced hydrophobic interactions within N-terminal elements and between N- and C-terminal ends are responsible for the improved thermostability of Xyn10A_ASPNG.

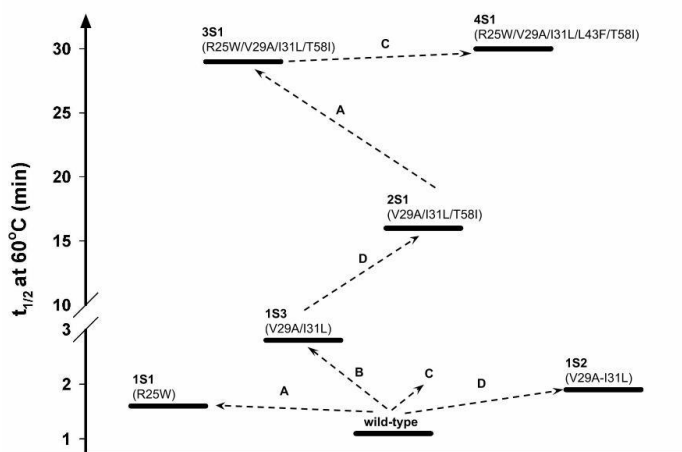


Fig 1. Stepwise increase in Xyn10A_ASPNG thermostability ($t_{1/2}$ 60°C) along with the process of iterative saturation mutagenesis. The letters A, B, C and D correspond to saturation mutagenesis randomized at position 25, 29/31, 43 and 58, respectively. The best hits arising from each mutant library were indicated in the figure.

Development of mannuronan C-5 epimerases to perform *in vitro* tailoring and upgrading of alginates

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Alginate is a linear polysaccharide composed of β -D-mannuronic acid (M) and α -L-guluronic acid (G) 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 mannuronic acid residues (M) in the alginate chain to guluronic acid residues (G). These epimerases consist of two types of structural modules, A-module which contains the catalytic site of the enzyme and R-module which is involved in substrate and calcium binding, increasing the activity of the enzyme. Through the engineering of new hybrid mannuronan C-5 epimerases constituted by the A-module from algE6 and the R-module from algE4, new and different epimerization patterns are observed. Defined manipulation with the sequences of new enzymes and modifying the linkage zone between the A- and R- module of these innovative epimerases lead to a better comprehension of the 'mode of action' of the epimerases and open up greater avenues for developing more efficient epimerases that can be used to generate new alginate structures of relevant interest for different applications.

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Using alginate milk protein complexes for model foods to investigate how food structure affects satiety

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Obesity is an increasing problem in modern society. Food designed for weight management usually has reduced energy content as compared to their conventional counterparts. The structure of food may increase intraluminal viscosity affecting subjective satiety and energy intake. (1) Here it is hypothesized that foods with similar energy content (isoenergetic) but with different structures can have different influence on satiety. To investigate this hypothesis, the macromolecular interactions of pure whey proteins and alginate will be characterized using Isothermal Titration Calorimetry, Differential Scanning Calorimetry, Surface Plasmon Resonance, Size Exclusion Chromatography and Dynamic Light Scattering. Alginate is an indigestible polysaccharide composed of (1→4)-β-D-mannuronic acid and α-L-guluronic acid (2) that is extensively used in the food and pharmaceuticals industry for its gelling and stabilising properties, as well as its high viscosity. (3;4) The results obtained will be used to design model foods that will be tested by *in vitro* digestion. The goal is to create a toolbox of very well characterized isoenergetic model foods both in terms of the macromolecular interactions in the food matrix, texture and microstructure as well as *in vitro* digestion pattern. These model foods will then subsequently be used in the project StrucSat (<http://strucsat.ku.dk/>) to clarify how food structure *per se* influences satiety and energy uptake.

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Structural enzymology and engineering of β -mannanases and α -galactosidases for galactomannan modification

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β -Mannans are abundant, notably present as the major soft-wood hemicellulose (galactoglucomannan, GGM) which can be isolated from industrial sidestreams (1). Another β -mannan is dietary galactomannan (GM) from guar applied as a thickener in food. We are studying the structure, function and applications of enzymes that are responsible for the hydrolysis of GGM and GM (2, 3, 4, 5). These enzymes are the retaining glycoside hydrolases (GHs) α -galactosidase and β -mannanase. The enzymes in the study originate from different environments such as soil bacteria and fungi, marine organisms and human gut bacteria. The different origins and cellular locations reflect the enzyme function (e.g. mode of attack, heteroglycan recognition and product profiles). We have recently discovered and characterized β -mannanases and α -galactosidases from gut bacteria. A cell-attached GH family 26 β -mannanase from *Bifidobacterium adolescentis* carries a GM-binding carbohydrate-binding module (CBM) of family 23 (5). The abundance of bifidobacteria increased in mice and rats fed with partially hydrolysed GM, showing the bifidogenic effect of this type of processed dietary fiber (6, 7). A loop deletion in a GH36 α -galactosidase appear to be responsible for the unusually efficient hydrolysis of galactose substituents from GM. This is potentially applicable to alter the properties of mannan-based hydrogels (8) for drug-delivery applications.

β -Mannanase catalysed transglycosylation reactions can also be used to modify β -mannans. The transglycosylation efficiency, however, varies between homologous β -mannanases of the TIM-barrel clan GH-A. Certain β -mannanases are efficient in synthesising alkyl glycoside conjugates (9) with potential applications as environmentally friendly tensides. Our investigations include structure-based protein engineering to alter the functionality of β -mannanases as well as the development of novel isotope labelling/mass spectrometry methods to analyse product formation (4). Systematic studies of clan GH-A β -mannanases show the importance and specificity of acceptor binding, notably in the the +2 subsite. We are thus determining the structural characteristics for acceptor-binding of β -mannanases which are efficient in synthesising alkyl glycosides and others that are efficient in the synthesis of oligosaccharides (9) potentially applicable as prebiotics. Ongoing work focus on protein engineering of β -mannanases for specific synthetic applications.

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Structural and biochemical studies of sugar beet α -glucosidase exhibiting high specificity for long-chain substrates

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α -Glucosidase is an exo-type enzyme catalyzing the hydrolysis of α -glucosidic linkage at the non-reducing termini of substrate molecules. A majority of α -glucosidases exhibits preference for disaccharides and trisaccharides as substrates. In contrast, several plant α -glucosidases are known to display high substrate specificity for long-chain substrates. Among them, an α -glucosidase from sugar beet exhibits low K_m and high k_{cat}/K_m for maltoheptaose and soluble starch, even though the active site structure of the enzyme is typical pocket shape as well as other α -glucosidases [1]. To comprehend the binding of long-chain substrates to sugar beet α -glucosidase, a series of long-chain pseudo-saccharides, acarviosyl-maltooligosaccharides (AC5–AC10, where the numerals represent DP), were employed. AC5–AC10 have longer maltooligosaccharide parts than the maltose unit of acarbose (AC4), which is a transition-state analog for α -glucosidases. We had synthesized AC5–AC10 from AC4 and maltotetraose using a disproportionating enzyme [2]. In the current study, the potential of the acarviosyl-maltooligosaccharides for the inhibition of sugar beet α -glucosidase was evaluated, and their complex structure analysis was performed [3]. Kinetic analyses showed that AC4–AC10 were competitive inhibitors for sugar beet α -glucosidase and their K_i values were decreased with increasing in DP of the inhibitor. The plots of $\log K_i$ for AC4–AC7 against $\log(K_m/k_{cat})$ for the hydrolysis of maltotetraose–maltoheptaose showed linear correlation with correlation coefficient of $r=0.96$, suggesting that the acarviosyl-maltooligosaccharides mimic the transition state as well as AC4. The complex structure of sugar beet α -glucosidase bound with AC8 was determined at 1.5 Å resolution. The electron density of AC8 was observed only at the active site, which was formed by a long loop (N-loop) protruding from the N-terminal sandwich domain and the catalytic (β/α)₈-barrel domain containing two short insertions, subdomain b1 and b2. Two residues of AC8 at the non-reducing end were buried in the active-site pocket, while the maltohexaose part of AC8 at the reducing side attached to the surface of the N-loop and subdomain b2. The substrate binding at a distance from the active-site pocket was maintained largely by van der Waals interactions, with the five glucose residues at the reducing terminus retaining a left-handed single-helical conformation. These results indicate that sugar beet α -glucosidase ingeniously uses the self-stabilizing property of amylose and soluble starch to form stable Michaelis complexes, which is responsible for lower K_m and higher k_{cat}/K_m for these long-chain substrates.

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Transcriptional and functional analysis of polysaccharide utilization loci reveals novel mechanisms of carbohydrate foraging by uncultivated gut bacteria

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Humans rely on their symbiotic microorganisms to provide the enzyme machinery required for the breakdown of complex glycans, mostly provided by dietary fibers. In particular, to face the structural complexity of plant cell wall polysaccharides, gut bacteria have evolved sophisticated strategies consisting of gene clusters encoding for multi-functional enzyme complexes. The first extensive activity-based metagenomics study focused on CAZyme discovery from the human gut microbiome revealed that numerous uncultivated bacteria, which represent more than 70% of the gut microbiota, possess a very complete glycan degrading enzymatic arsenal [1]. Involving mostly glycoside hydrolases, it is encoded by various multigenic clusters acquired by horizontal gene transfers, and annotated as putative Polysaccharide Utilisation Loci (PULs).

In order to exploit these novel catabolic pathways for biorefinery and synthetic biology, a PUL functional characterization strategy was developed, based on their rational engineering, metabolic and transcriptional analysis in *E. coli*. This approach was applied to the characterization of a multigenic system encoding for putative proteins involved in the binding, the degradation and the import of prebiotic xylo-oligosaccharides (XOS) [2] by an uncultivated bacterium assigned to the *Bacteroides* genus. Transcriptional analyses revealed that in *E. coli*, these genes are not transcribed as a polycistronic mRNA. Moreover, a series of deletions was generated to investigate function of the different components of the PUL specific of XOS catabolism. In particular, this work has demonstrated the specificity of a novel carbohydrate transporter for XOS of polymerization degree up to 4.

This strategy allows circumventing the difficulties of the PUL characterization in native strains and provides a promising framework to better understand their component functionalities, especially those of carbohydrate transporters, which are key elements of catabolic pathways.

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Is the metabolic preference for specific β -galactosides established by enzymes or by uptake systems in gut adapted bacteria?

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A diversity of β -galactosides of different monosaccharide composition and glycosidic linkages is abundantly present in human diet, e.g. in fruits, vegetables and milk (1, 2). With the exception of lactose, β -galactosides are indigestible by human enzymes presenting an attractive nutritional resource to human gut adapted bacteria. Oligomeric β -galactosides are reported to preferentially stimulate the growth of health promoting members of the gut microbiota, especially those from the *Bifidobacterium* genus. A degree of species-specific β -galactosides utilization by bifidobacteria is observed, but the possible contribution of transport systems to this remains unexplored.

A β -galactoside utilization gene cluster from *Bifidobacterium animalis* subsp. *lactis* BI-04 encodes a transcriptional regulator, an ATP-binding cassette (ABC) importer and an intracellular β -galactosidase of glycoside hydrolase family 42 (GH42) (3). The latter enzyme has been shown to exhibit broad specificity hydrolyzing β -1,6, β -1,3 and β -1,4 galactosidic bonds with only a modest preference for β -1,6 bonds (4). The objective of the present study is to investigate the ligand preference of the solute binding protein associated with the ABC transporter responsible for β -galactosides uptake in this locus.

The results of the present study represent a unique case allowing the comparison of selectivities of a transport protein and a glycoside hydrolase presumably involved in the metabolism of the same glycan type. These findings will be presented and discussed to highlight the role of ABC-mediated glycan transport in establishing the metabolic preference in an important group of the gut microbiota.

Acknowledgements

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Chitin hydrolysis by *Chitinbacter tainanensis* enhancing via explosive puffing

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The purpose of this study is to investigate enhancement of chitin hydrolyzed to N-acetyl-D-glucosamine by *Chitinbacter tainanensis* using explosive puffing to modify structure of chitin. The raw and puffed chitins were prepared and were used as substrate for *C. tainanensis* to study the influence of molecular chain and spatial structure of chitin on degradation. Chitin was tempered to 75%, 50% and 35% moisture content and equilibrated at 25°C then being puffed in a puffing gun. The structure of chitin was modified by explosive puffing which were analyzed by XRD, SEM and density. The bulk densities of puffed chitins were significantly decreased with the decreased of puffed moisture content. The crystal structure of puffed chitin was strongly destroyed with lower moisture content. The holes on surface of puffed chitins were became larger and more with the decreased of puffed moisture content. The puffed chitin was more easily utilized by *C. tainanensis* than the unpuffed chitin. The *C. tainanensis* cultivated in puffed chitin with 75% moisture content had the highest yield of N-acetyl-D-glucosamine after 72 hours.

Table 1. The density, crystallinity index and yield of hydrolysis (Brix, %) of puffed chitin with different moisture contents

Chitin	Tapped density (g/cm ³)	Crystallinity index (CrI ₀₂₀ , %)	Brix (%)
Raw	0.1806	78.76	0.9
75%	0.1686	77.09	1.4
50%	0.1572	72.40	1.1
35%	0.1504	69.57	1.0

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Supressing transglycosylation to improve hydrolysis of cellobiose to glucose

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The surface properties of the active site chamber or cleft are important factors in shaping the kinetic behavior of enzymes. We showed earlier that changing the shape of active site can improve the reaction of xylose isomerase with a promiscuous substrate, L-arabinose (Karimäki et al., 2004). Not unexpectedly, the mutated active site side chain can also impose via a water link its effect on stabilizing another promiscuous substrate, L-ribose, into the active site of xylose isomerase, and thus improving the reaction rate (Santa et al., 2005). The kinetic properties, reaction balance and product pattern have a key role in determining the efficiency of multienzyme reaction series, as in the hydrolysis starch and cellulose and in synthetic biological reaction pathways.

In cellulose hydrolysis to glucose, especially in high substrate and product concentrations, the end-product inhibition can be a significant factor in determining the product yield. Also other concentration-dependent factors than end-product inhibition, affect the yield. High substrate concentration is known to trigger the reverse reaction, transglycosylation, in glycoside hydrolases. This behavior is utilized biotechnologically to produce galacto-oligosaccharides from lactose to be used in food applications. In the hydrolysis of starch and cellulose to glucose such property of hydrolytic enzymes decreases the yield (e.g., Bohlin et al., 2013).

Beta glycosidase (BGAL_SULAC) from *Sulfolobus acidocaldarius* is highly thermostable enzyme (optimum at 90-95°C with cellobiose as substrate) cleaving efficiently lactose and cellobiose. We studied how the active site mutations affect the kinetic behavior of the enzyme (Anbarasan et al., 2015) and observed an effect on the formation of trisaccharide in high concentration of cellobiose. When cellobiose was the substrate in 200 g/L concentration, BGAL_SULAC produced 40-60% amount of trisaccharide (probably cellotriose) in relation to produced glucose. A mutation in the active site close to the catalytic residues reduced remarkably the trisaccharide formation, and therefore, increased the glucose yield.

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Evaluation of the secretomes of cellulolytic and chitinolytic microorganisms

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Cellulose and chitin are the two most abundant biopolymers in nature and several microorganisms are known to degrade these biopolymers (e.g. [1, 2]). Investigation of proteins secreted by microorganisms growing on different chitin or cellulose substrates potentially allows identification of novel proteins involved in biomass degradation. We use here a novel agar-plate method that enables isolation of secretomes with low contamination of intracellular proteins.

Microorganisms were grown on agar-plates with a filter included in the center of the plate. Different chitin or cellulose substrates were used as the sole carbon source and proteins were isolated, followed by trypsination and identification by bottom-up mass spectrometry using a QExactive (Thermo) coupled to a nanoHPLC (Dionex). Relative protein abundances were calculated using label-free quantification with the MaxQuant software.

Several carbohydrate-active enzymes (CAZymes) were identified, including substrate specific enzymes like chitinases and cellulases. In addition, we found several uncharacterized proteins, which provide leads towards the identification of hitherto non-identified elements in the enzymatic machineries used by microorganisms when degrading different biomasses.

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Functional metagenomics boosts enzyme discovery for plant cell wall polymer breakdown

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To face the structural diversity of plant polysaccharides and polyaromatic compounds composing their main carbon source in many ecosystems, microorganisms have evolved sophisticated multi-functional enzyme complexes. In order to unlock them from the vast world of uncultured microorganisms, and to explore their extraordinary functional potential, a high-throughput function-based metagenomic approach was developed to boost the discovery of enzymes involved in complex polymer breakdown.

Metagenomic libraries from the highly diverse bovine rumen microbiome, totalising 1.5 Gbp of DNA, were screened for glycoside-hydrolase, esterase, and oxidase activities. Several hundreds of positive clones were found active on the screening substrates. In a second step, positive clones were characterised for their abilities to degrade resistant plant cell wall substrates. The metagenomic DNA of the best performing clones were sequenced using the MiSeq technology, and thus functionally and taxonomically annotated, highlighting very original sequences, including novel CAZy modules.

This work allowed the identification of dozens of novel enzymes of high potential for numerous biotechnological processes, as they are highly efficient, alone or acting synergically, to breakdown plant cell wall polysaccharides and polyaromatic compounds.

Oligosaccharides production using a glucansucrase from a lactic acid bacteria strain in its free and immobilized form

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Glucansucrases, also known as glucosyltransferases (GTFs) are extracellular enzymes that synthesize high molecular weight glucans or in the presence of acceptors molecules, low molecular weight oligosaccharides, using sucrose as a donor. In the present study, a glucansucrase from a lactic acid bacteria strain was applied at highly concentrated sucrose solutions. Reaction parameters were optimized and the synthesized oligosaccharides were quantified and partially characterized. Glycosidic linkages were analysed in the crude reaction mixtures using DOSY NMR. The enzyme was used in its free (soluble) and immobilized form, after its immobilization into a polyvinylalcohol matrix in the form of LentiKat's.

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Factors affecting enzymatic cellulose hydrolysis in ionic liquid solutions

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Certain ionic liquids (ILs, salts with melting points < 100 °C) dissolve cellulose and in some cases even wood [1]. It has been shown that pretreating lignocellulose with ILs increases the feedstock's digestibility in enzymatic hydrolysis. However, the cellulases typically used for cellulose hydrolysis are sensitive to inactivation by even low amounts of IL, and therefore a water-intensive washing step is needed between the IL pretreatment and hydrolysis steps. Developing compatible cellulase-IL systems for one-pot hydrolysis is an intensively studied topic, but achieving this objective exerts a need to elucidate the factors governing IL-induced cellulase inactivation in detail, which is the topic of this work.

The hydrolytic action of *Trichoderma reesei* Cel5A was compared in 20 % of different ILs during 72 h hydrolysis at 45 °C using 1 % microcrystalline cellulose as substrate [2]. The hydrolysis yields were below 2.5 %, compared to 7.7 % in the buffer reference system. The differences in hydrolysis yields were generally small between the different ILs. In another experiment, the endoglucanase stability of a *T. reesei* cellulase cocktail was measured in two pure ILs, 1-ethyl-3-methylimidazolium acetate ([EMIM]AcO) and 1,3-dimethylimidazolium dimethylphosphate ([DMIM]DMP) [3]. In [EMIM]AcO, complete inactivation was observed in 4 h whereas the endoglucanase activity decreased only slowly in [DMIM]DMP. This result suggests that cellulase inactivation in IL solutions may not be the only reason for observed low hydrolysis yields, as hydrolysis yields in 20 % [EMIM]AcO and [DMIM]DMP were similar, but the endoglucanase stability differed greatly in the two ILs. The effect of [EMIM]AcO and [DMIM]DMP on cellulase substrate binding and the role of the carbohydrate-binding module (CBM) were also studied using the intact (core domain + CBM) and core domain (lacking CBM) proteins of *T. reesei* Cel5A and Cel7A [4]. The results showed increasing IL concentrations to inhibit substrate binding and especially binding through the CBM was affected.

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Structural-functional analysis reveals a specific domain organization in family GH20 hexosaminidases

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GH20 hexosaminidases are widely distributed in nature catalyzing the hydrolysis of *N*-acetyl-hexosaminyl residues in oligosaccharides and glycoconjugates. They present several accompanying domains with a diverse organization [1]. We performed structural and sequence analysis of the known GH20 enzyme structures that lead us to propose two different domain organization models: model A, containing at least a non-catalytic GH20b domain and the catalytic GH20 always followed by an extra alpha-helix, and model B, with only the catalytic GH20. In addition, the GH20b-GH20- α topology of model A appeared not always to be the minimal functional unit. We see that for GH20 model A enzymes, the substrate-binding cavity always involved a remote element which is provided by a long loop of the catalytic domain, or, when the loop is short, by an accompanying domain of the same protein or of a dimer partner.

The large *B. bifidum* lacto-*N*-biosidase (LnbB) was used as a model protein due to its biotechnological interest and structural complexity [2,3]. LnbB enzyme is involved in the degradation of human milk oligosaccharides, specifically the hydrolysis of tetrasaccharide Gal β 1,3GlcNAc β 1,3Gal β 1,4Glc to lacto-*N*-biose (Gal β 1,3GlcNAc) and lactose. It consists of five domains GH20b-GH20-lectin-CBM32-Iglike and the truncated protein GH20b-GH20- α proved not to be active. Here we report evidences of the requirement of a remote element for the protein to be catalytically active. *In vitro* complementation experiments of the inactive truncated protein and the C-terminal lectin domain restores activity due to protein-protein interactions that locates the remote element in the active conformation. This analysis provides new insights for further enzyme engineering for biotechnological applications.

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Novel carbohydrate targeting mechanisms by the human gut symbiont *Bacteroides thetaiotaomicron*

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Over 30% of our resident gut microbiota are from the *Bacteroidetes* family (1). These gram-negative, obligate anaerobes are well armored with a repertoire of carbohydrate degrading enzymes to battle the gut environment for competing carbohydrate nutritional resources. One species, *Bacteroides thetaiotaomicron*, devotes 10% of its genome to carbohydrate foraging. Discrete loci within its genome are upregulated in response to carbohydrates dependent on the source (2). These sources arise from either dietary (eg: plant polysaccharide) or mammalian (eg: mucins) origin. Using a combination of techniques we have discovered novel aspects of *B. thetaiotaomicron* degradation of unique polysaccharide sources. By combining anaerobic growth, cultivation, gene expression, mass spectrometry and enzymatic characterization techniques, we are able to view overall novel carbohydrate degradation mechanisms and identify new mechanisms which are responsible for making *B. thetaiotaomicron* a prominent and well established human gut symbiont.

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Insight into structural, biochemical and *in silico* determinants of ligand binding specificity of family 6 carbohydrate binding module (CtCBM6) from *Clostridium thermocellum*

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CtCBM6 is a part of modular glucuronoxylan-xylanohydrolase (CtXynGH30) from *Clostridium thermocellum* [1-2]. CtCBM6 was cloned, expressed and purified as a soluble ~14 kDa protein. CtCBM6 showed binding with polysaccharides containing xylan main chain as well as decorated with arabinose or glucuronic acid as side chains. Quantitative binding analysis with soluble polysaccharides by affinity electrophoresis and ITC revealed that CtCBM6 displays higher affinity towards wheat- and rye-arabinoxylans than beechwood-, birchwood- and oat spelt-xylan. Site-directed mutagenesis revealed that Tyr28 and Phe84 in Cleft A play major role in ligand binding. Protein melting study displayed thermostable nature of CtCBM6 and Ca²⁺ ions did not affect its structure stability and binding affinity. CtCBM6 model was developed by homology modelling and refined by molecular dynamic simulation. Structure analysis indicated the presence of predominantly β -sheets forming a jelly roll fold. CtCBM6 displayed two potential binding clefts. Cleft A, is situated between two loops connecting β 4 to β 5 and β 8 to β 9 strands. Tyr28 and Phe84 present on these loops made a planar hydrophobic ligand binding surface to accommodate flat sugar ring. Cleft B, is located on the concave surface of β -sandwich fold. Tyr34 and Tyr104 make a planar hydrophobic platform, inaccessible to ligand due to hindrance by Pro68. CD spectrum of CtCBM6 showed only β -strands and random coils and supported *in silico* structure prediction.

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Diversity in β -galactosidase specificities within *Bifidobacterium*: towards an understanding of β -galactoside metabolism in the gut niche

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The *Bifidobacterium* genus harbors several health promoting members of the gut microbiota, which display metabolic specialization by preferentially utilizing dietary or host-derived β -galactosides. To approach a deeper understanding of the β -galactoside catabolism in the gut niche, we investigated the bioinformatic, biochemical and structural properties of glycoside hydrolase family 42 (GH42) β -galactosidases within *Bifidobacterium* associated with the human gut. GH42 β -galactosidases segregate according to function in a phylogenetic analysis (Fig. 1) and display a large variety of sub-specificities (1–3) in accordance with the diversity and complexity of β -galactosides available in the gut. The first function-structure insights in GH42 illustrate that diversity manifested in sub-specificities, correlates to subtle changes in loop regions in the near vicinity of the site of catalysis (1). We are currently pursuing further studies in sub-specificities and structures to advance the understanding of the evolution of GH42 that at the molecular level support metabolic specialization in the gut niche.

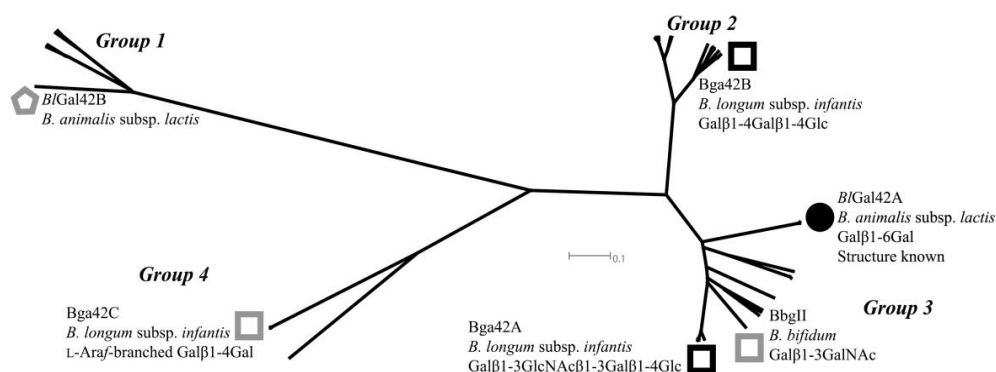


Fig 1. Phylogenetic relationship of GH42 β -galactosidases within *Bifidobacterium*

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Mining anaerobic digester consortia metagenomes for secreted carbohydrate active enzymes

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Anaerobic digesters (ADs) are one of several ways to produce renewable energy, which in the case of ADs is in the form of methane. Several microbial groups are involved in anaerobic degradation of organic wastes such as animal manures and wastewater, and solid organic wastes such as sludge, crop, and food wastes (Alvarado *et al.*, 2014).

The processes and the roles of the microorganisms that are involved in biomass conversion and methane production in ADs are still not fully understood. We are investigating thermophilic and mesophilic ADs that use wastewater surplus sludge for methane production. To gain insight into both the degradation of the carbohydrates and the various roles of the microbes in the ADs we have mined metagenomes from both types of ADs for glycoside hydrolases, carbohydrate esterases, polysaccharide lyases, auxiliary activities, and carbohydrate binding modules. The mining was done with the Peptide Pattern Recognition (PPR) program (Busk and Lange, 2013), which is a novel non-alignment based approach that can predict function of *e.g.* CAZymes. PPR identifies a set of short conserved sequences, which can be used as a finger print when mining genomes for novel enzymes. In both thermophilic and mesophilic ADs a wide variety of carbohydrate active enzyme functions were discovered in the metagenomic sequencing of the microbial consortia. The most dominating type of glycoside hydrolases were β -glucosidases (up to 27%), α -amylases (up to 10%), α -glucosidases (up to 8%), α -galactosidases (up to 9%) and β -galactosidases (up to 7%). For carbohydrate esterases the by far most dominating type was acetylxylan esterases (up to 59%) followed by feruloyl esterases (up to 16%). Less than 15 polysaccharide lyases were identified in the different metagenomes and not surprisingly no polysaccharide monooxygenases were identified. For the carbohydrate binding modules one of the most dominating families were CBM48 (up to 16%).

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This work is supported by the Villum Foundation.

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Structural and functional characterization of the *Clostridium perfringens* N-acetylmannosamine-6-phosphate 2-epimerase essential for the sialic acid salvage pathway

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Pathogenic bacteria are endowed with an arsenal of specialized enzymes to convert nutrient compounds from their cell hosts. The essential N-acetylmannosamine-6-phosphate 2-epimerase (NanE) belongs to a convergent glycolytic pathway for utilization of the three amino sugars, GlcNAc, ManNAc and sialic acid. The crystal structure of ligand-free NanE from *Clostridium perfringens* reveals a modified TIM (β/α)₈ barrel in which a stable dimer is formed by exchanging the C-terminal helix. By retaining catalytic activity in the crystalline state, the structure of the enzyme bound to the GlcNAc-6P product identifies the topology of the active site pocket and points to invariant residue Lys66 as a putative single catalyst, supported by the structure of the catalytically inactive Lys66Ala mutant in complex with substrate ManNAc-6P. ¹H-NMR-based time course assays of native NanE and mutated variants demonstrate the essential role of Lys66 for the epimerization reaction with participation of neighboring Arg43, Asp126 and Glu180 residues. These findings unveil a one-base catalytic mechanism of C2 deprotonation/reprotonation via an enolate intermediate and provide the structural basis for the development of new antimicrobial agents against this family of bacterial 2-epimerases.

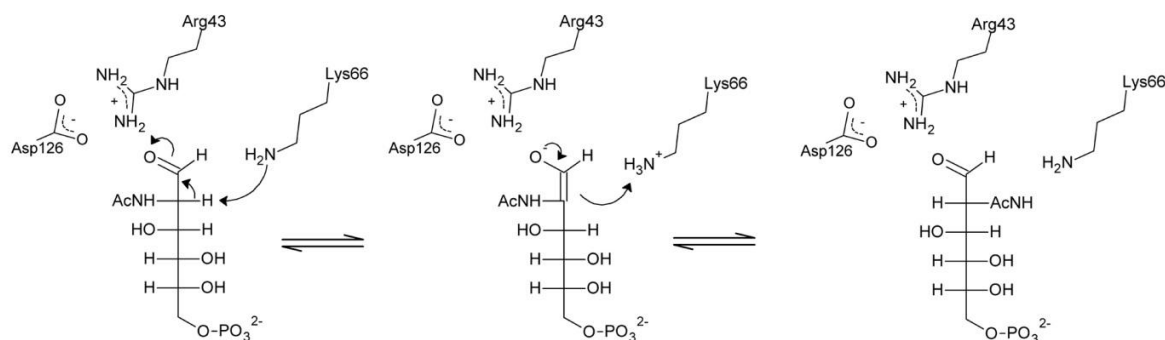


Fig 1. Once the C2 proton is abstracted by the base catalyst Lys66, a negatively charged intermediate is stabilized by the salt bridge and Glu180. A proton is donated by the same Lys66 catalyst to the enolate intermediate to yield the GlcNAc-6P product.

Discovering novel glycan utilization loci in probiotic bacteria

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Consumption of host-indigestible glycans is essential for the maintenance of a healthy population of gut microbes. The thriving of probiotic bacteria, *Bifidobacterium* and *Lactobacillus* being the most commercially exploited strains, necessitates the intake of prebiotics¹. Mannan-oligosaccharides (MOS) are one such potential prebiotic which is present in nature in different structures such as galactomannan and glucomannan². Xyloglucan utilization loci have also recently been identified and characterized in several *Bacteroidetes* strains³. Not much is known about either MOS or xyloglucan utilization by probiotics, even less on their roles as prebiotics.

Identification of putative loci for MOS or xyloglucan utilization has been pursued through genome-mining in different strains of probiotic bacteria. Utilization of either glycan will be investigated in these probiotic taxa through bacterial growth assays. Identification of upregulated genes when the bacteria grow in media with or without oligosaccharides, including novel glycoside hydrolases (GHs) genes, will be carried out through transcriptomics and proteomics. These identified GHs will be functionally characterized including crystal structure determination. This investigation will broaden the knowledge of the different strategies gut microbes have evolved in the crowded and competitive gut environment to utilize varying carbohydrate sources. Preliminary results will be presented.

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This research project is supported by the Sino-Danish Center (SDC) and Technical University of Denmark (DTU)

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Activity-based probing of α -L-fucosidase

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α -L-Fucosidases (GH29, GH95) catalyse the hydrolysis of fucose from fucosylated glycans in an exo-fashion. GH29 fucosidases are medically relevant due to, for example, the differential regulation they have in a number of cancers (they have been shown to be potent biomarkers for a number of cancer types¹), and links they have to the virulence of pathogens such as *Helicobacter pylori*.² Deficiency in GH29 activity also causes the severe lysosomal storage disorder fucosidosis.³

Activity-based probes (ABPs) exploit the catalytic mechanisms of enzymes to allow the specific trapping of active enzymes by the formation of irreversible covalent ABP complexes.⁴ Herein we report the use of ABPs for probing α -L-fucosidases (**Fig 1.**), allowing quantification of α -L-fucosidase activity levels *in vitro* and *in vivo*, and prove covalent attachment of an ABP scaffold to a bacterial α -L-fucosidase through X-ray crystal structure.

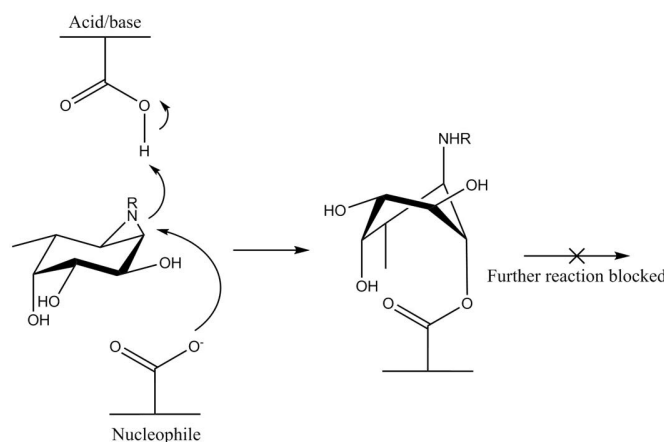


Fig 1. Schematic for covalent trapping of α -L-fucosidase with an aziridine ABP.

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Gene synthesis, expression and characterization of a thermostable endo- β -1, 4-mannanase

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Thermostable mannanase ManAd3 from *Caldibacillus cellulovorans* was produced as codon optimized version in *Pichia pastoris* strain GS115 from pPIC9k vector (recombinant strain ManAhr-GS115). The protein was secreted to the culture medium as a sole visible protein with a molecular mass about 30 kDa as shown in SDS-PAGE. The enzymatic properties were determined and the optimal reaction temperature and pH were 75°C and pH 6.0, respectively, and the specific activity was up to 3200 U/mg. Moreover, the enzyme retained over 90% of its activity after 30 min treatment at 75°C, pH 6.0.

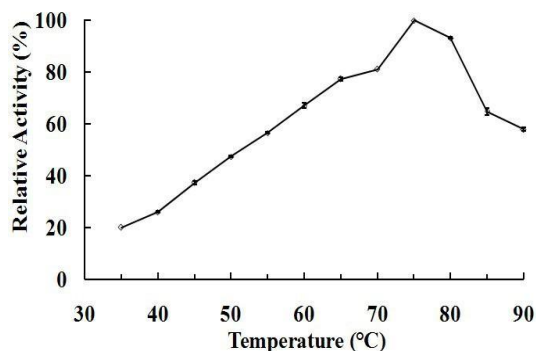


Fig 1. Temperature-dependent activity profile of mannanase ManAhr at pH 4.5 in 10-min assay.

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Expression a hyperthermostable *Thermotoga maritima* xylanase 10B in *Pichia pastoris* GS115 and its tolerance to ionic liquids

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The hyperthermostable xylanase 10B from *Thermotoga maritima* (TmXYN10B) was overexpressed as codon optimized in *Pichia pastoris* strain GS115. The expressed xylanase was about 40 kDa, and showed the optimal temperature at 100 °C in 60-min assay. Also, it showed 92% of maximal activity at 105 °C. The half life of inactivation was over 120 min at 100 °C. Thermostability is thought to protect against inactivation by biomass-dissolving ionic liquids. We tested how TmXYN10B tolerates [EMIM]OAc, [EMIM]DMP and [DBNH]OAc. At 80 °C, 15% ionic liquids caused about 25-45% inactivation and 35% ionic liquids about 74-86% inactivation. Although the activity loss in TmXYN10B by the ionic liquids was higher than in the most ionic liquid-tolerant xylanases, TmXYN10B was able to retain partial activity (49%) even at 90 °C in 35% [EMIM]DMP during 24-hour reaction. In the same conditions, the 15% [EMIM]DMP caused only 13% loss in the activity. In conclusion, when partial activity in quite long reactions at very high temperatures in aqueous ionic liquid solutions is needed, the TmXYN10B enzyme showed promising performance.

Tailor-made potato starch

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Starch, the most important storage carbohydrate, is widely used in food and non-food industry. Native starches have shortcomings that restricts the end uses, hence starch modification is a prerequisite for some industrial applications (Xu *et al.*, 2014). With a better understanding of starch biosynthesis and degradation pathways, new genetic tools have become available for the modification of starch composition and to produce starches with novel functionalities *in planta*. This approach has the enormous potential for tailoring starches with less energy expenditure and without pollutant chemical, thereby broaden starch applications in an economically and environmentally-friendly way. We developed the so-called Starch Binding Domain (SBD) technology to target effector proteins to starch granules during the biosynthesis process in potato (Ji *et al.*, 2003). By using this SBD technology, several heterologous proteins were successfully incorporated into starch granules and resulted in the modification of potato starches in the following aspects: (1) the morphology of starch granule; i.e., expression of an *E. coli* maltose acetyltransferase (MAT) (Nazarian Firouzabadi *et al.*, 2007), a truncated mutansucrase (GTFICAT) (Kok-Jacon *et al.*, 2005), an *E. coli* branching enzyme (GLGB) (Huang *et al.*, 2013); (2) the size of starch granule; i.e., expression of multiple appended SBDs (Ji *et al.*, 2004), a *Neisseria polysaccharea* amylosucrase (Huang *et al.*, 2014); (3) the phosphate content of starch granule; i.e., expression of human phosphatase (Iaforin). Moreover, the effect on starch compositions and properties were investigated. Ultimately, transcription profiling of genes in those transformants were analyzed to get better understanding of starch biogenesis in tuberous storage starch.

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Reconstruction of genome-scale metabolic model of *Brevibacillus thermoruber* 423 for design of improved EPS production strategies

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Microbial exopolysaccharides (EPSs) gain increasing importance as bioactive and biocompatible polymers in many industries. Despite the vast amount of reports on mesophilic EPS production mechanism, there is only limited knowledge on EPS biosynthesis mechanisms by thermophilic bacteria. In a recent study, owing to high EPS productivity, *Brevibacillus thermoruber* 423 is considered as a promising thermophilic model organism and cell factory for microbial EPS production [1] and using genomic information a hypothetical mechanism for EPS biosynthesis was generated (Fig 1) [2]. To gain more insight into the EPS biosynthesis mechanism of this thermophile, a genome-scale metabolic model was reconstructed and the model was validated by experimental data. The reconstruction process started from the annotated genome of *Brevibacillus thermoruber* 423 [2]. A collection of computational tools, including Pathway Tools, COBRA Toolbox is used to predict gene-reaction associations from annotated genome, to reconstruct the metabolic network based on genomic, biochemical and physiological information, to find and curate reaction gaps and finally to simulate the resulting metabolic network. The obtained model is further used to generate hypotheses on the optimal strategies for enhanced EPS production by *in silico* simulations using different optimization algorithms and objective functions (e.g. max growth rate, max ATP synthesis). The draft model contained 1798 reactions and 1981 metabolites, with 773 orphan reactions. The biomass definition is taken from *Bacillus subtilis* iYO844 model and used as template. Overall, this work presents genome-scale metabolic network of the EPS-producing thermophilic microorganism and offers strategies for enhanced EPS biosynthesis. These results are expected to accelerate the research on thermophilic bacteria using systems biology approaches and design of metabolic engineering strategies to enhance EPS production.

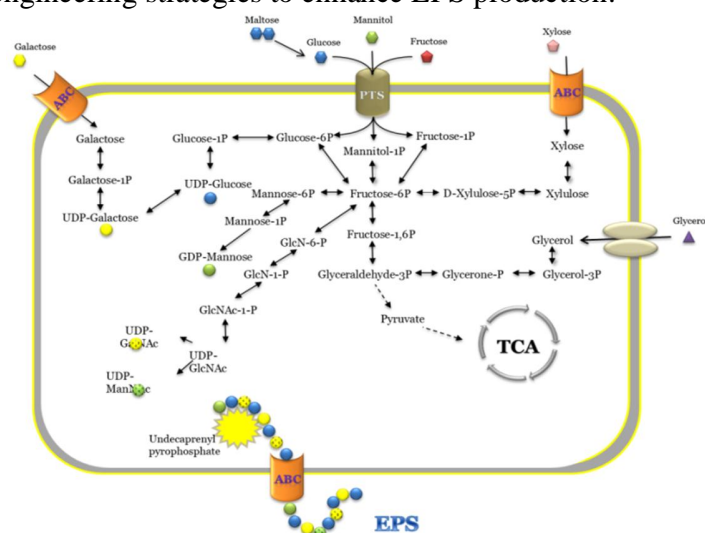


Fig 1. Proposed EPS biosynthesis mechanism of *B. thermoruber* 423 [2]

Acknowledgements The financial support of Marmara University through project FEN-C-DRP-110913-0380 is gratefully acknowledged.

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NMR spectroscopic methods in engineering of sugar acid pathways in yeast

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In our projects on engineering sugar acid related pathways, the structural analysis of the products by NMR has been a major task, because in addition to the pyranose and furanose ring structures, these molecules may also form lactone rings, and some of the molecules we have been interested in have also keto groups, potentially leading to enol type structures in some conditions¹. An additional challenge is the symmetry of molecules like mucic acid, in which cases positional ¹³C labelling has been used to turn the molecules unsymmetrical from NMR point of view.

The product of a sugar acid forming enzymatic reaction is often a lactone which is not very stable. Therefore, following the reaction in real time has proven an invaluable tool in identification of the initial product of the enzyme and the rate of its spontaneous opening^{1,2}. This method is also a way to follow enzyme kinetics and, using modern water suppression methods, the enzyme reaction can be carried out even in 100 % H₂O buffer (instead of the normal D₂O). This is important, when the water itself participates the reaction or if the enzyme preparation (a cell extract) has a low activity.

If the aimed product has several molecular forms that can spontaneously be converted to one another (like a lactone to an open chain sugar acid), it is of interest, whether this conversion takes place already intracellularly and which one of the forms is exported from the cells. Therefore, we have used *in vivo* ¹H NMR of engineered, overproducing yeasts to follow the production and exportation rates of xylonolactone and xylonic acid³. A bonus achieved by these experiments is that intracellular pH can also be determined, because the ¹H signals of the proton close to the carboxylic group respond to the pH. This is important data, because pH drop due to the acid production can often compromise the viability of the cells.

In addition, the so called ligand-based NMR methods on ligand-protein interactions can be very useful in identification of the binding geometry of the ligand to a particular enzyme⁴. These methods do not need labelled proteins and the protein concentration can be very low.

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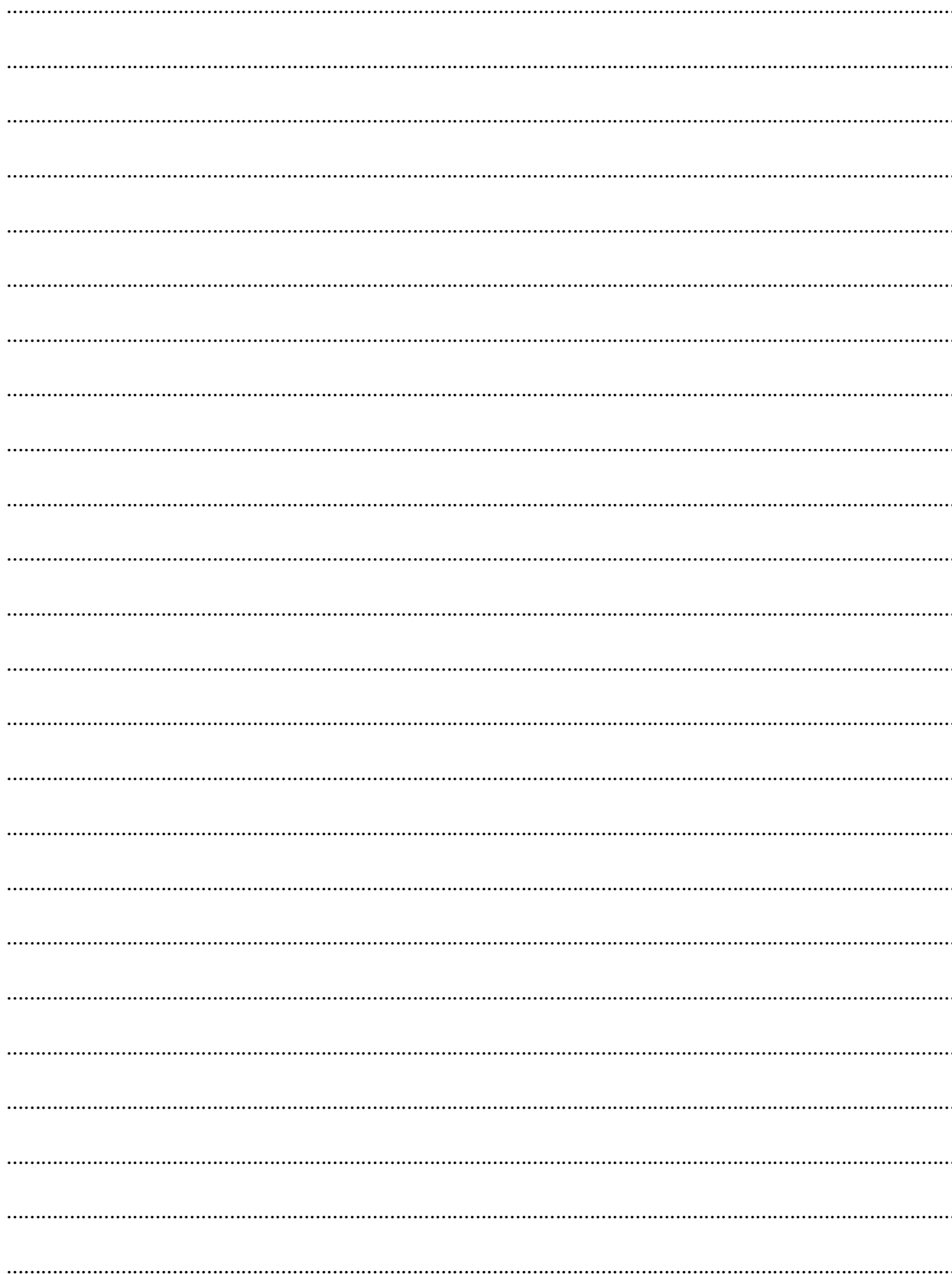
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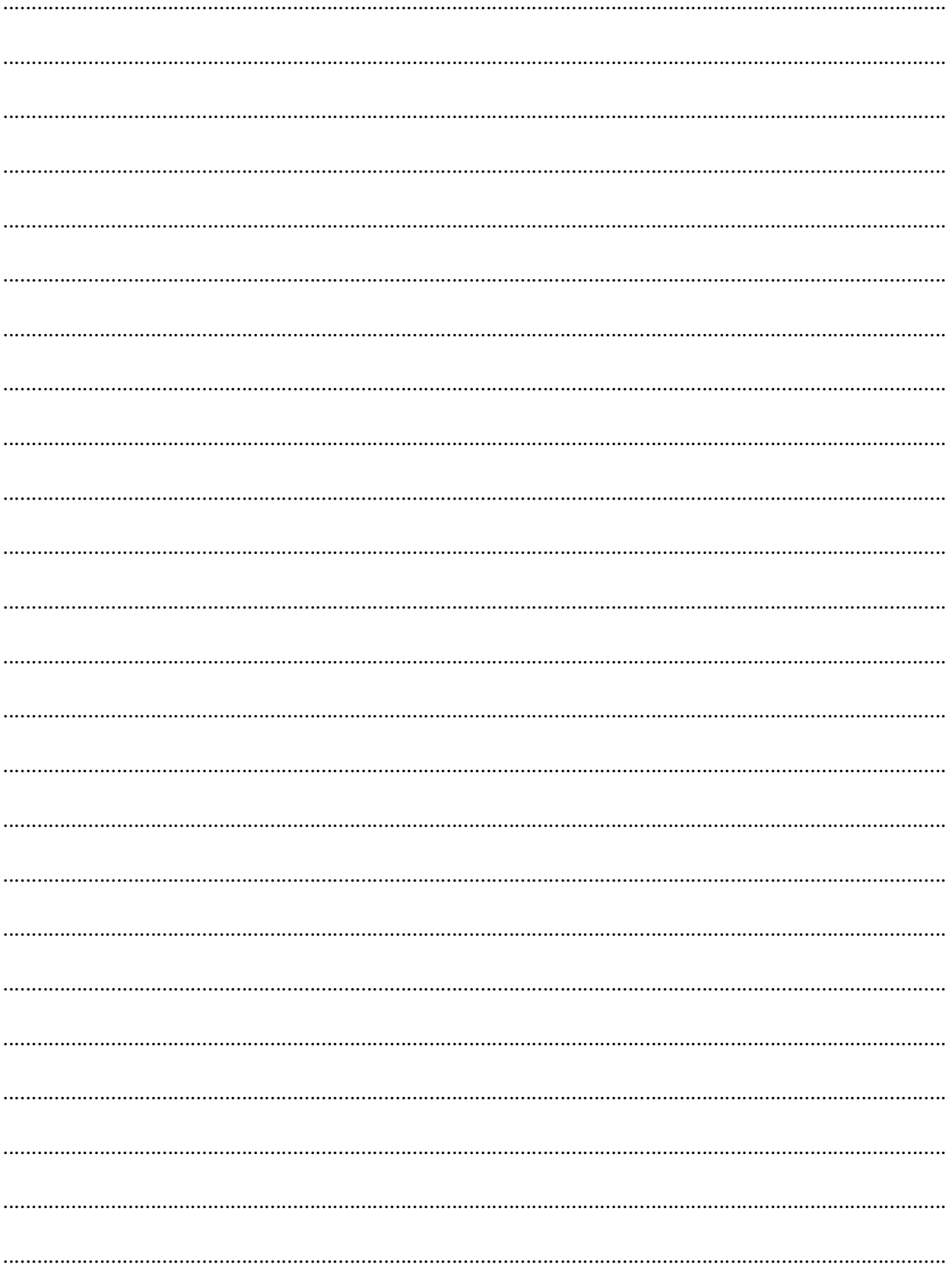
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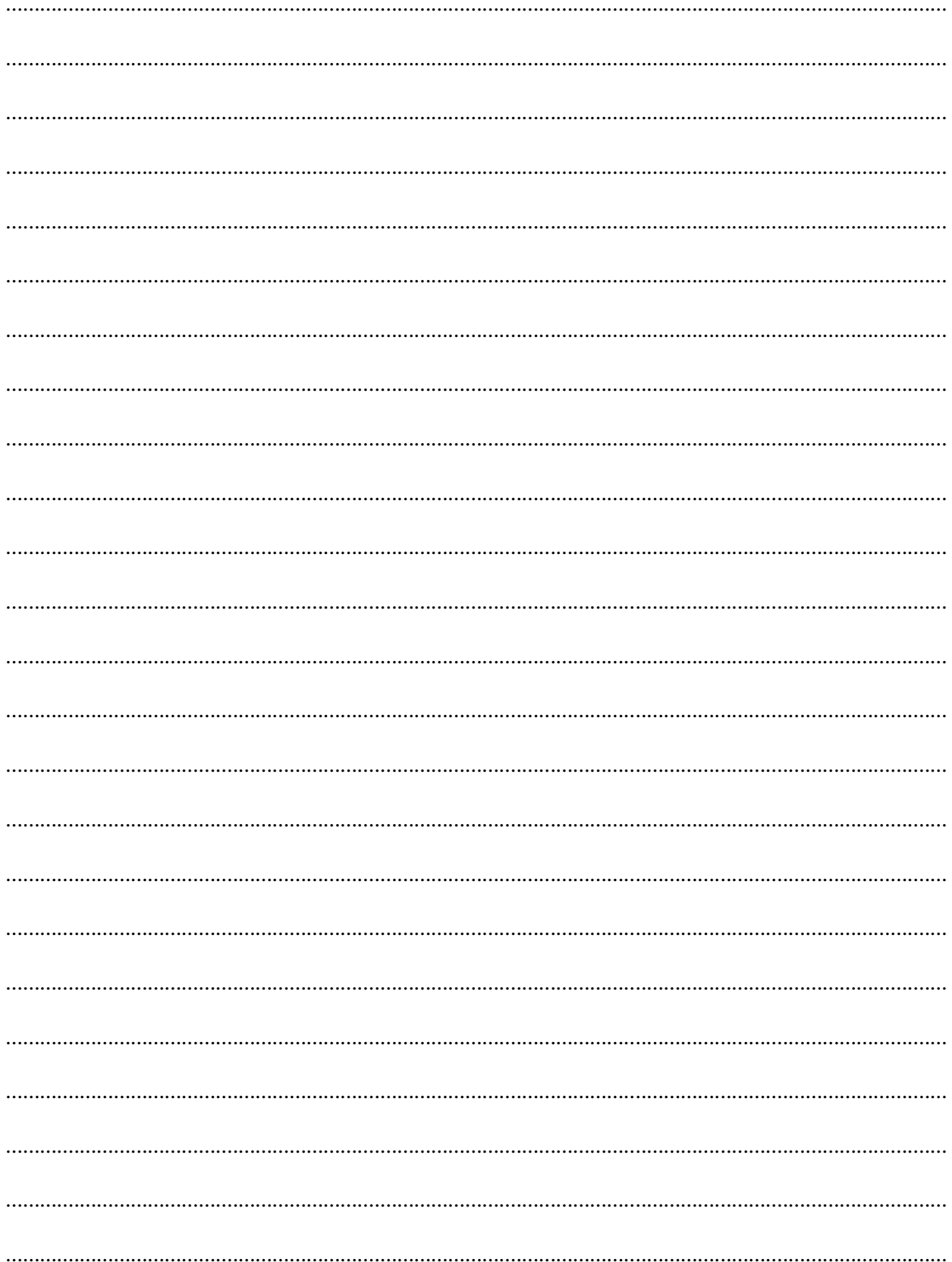
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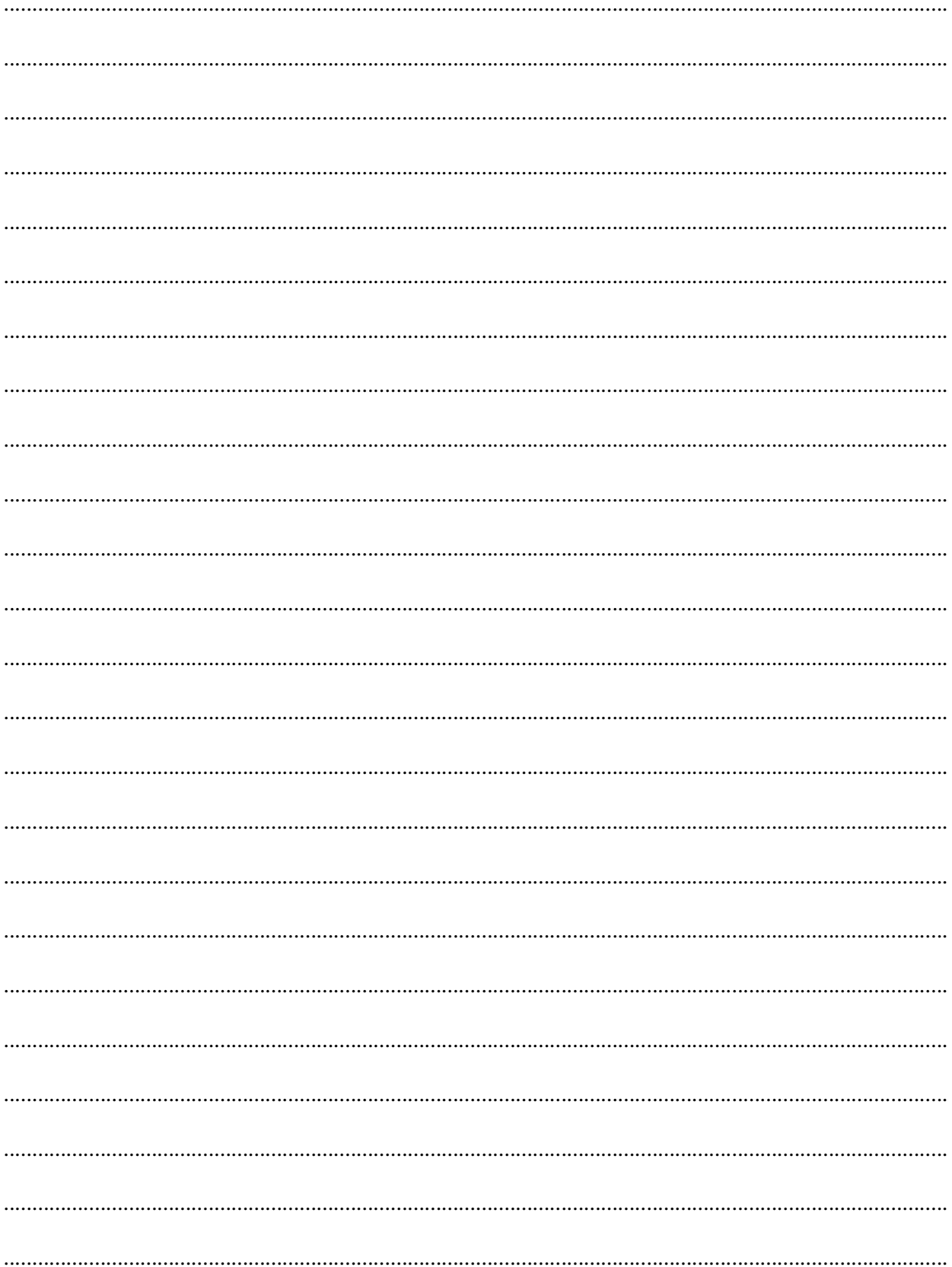
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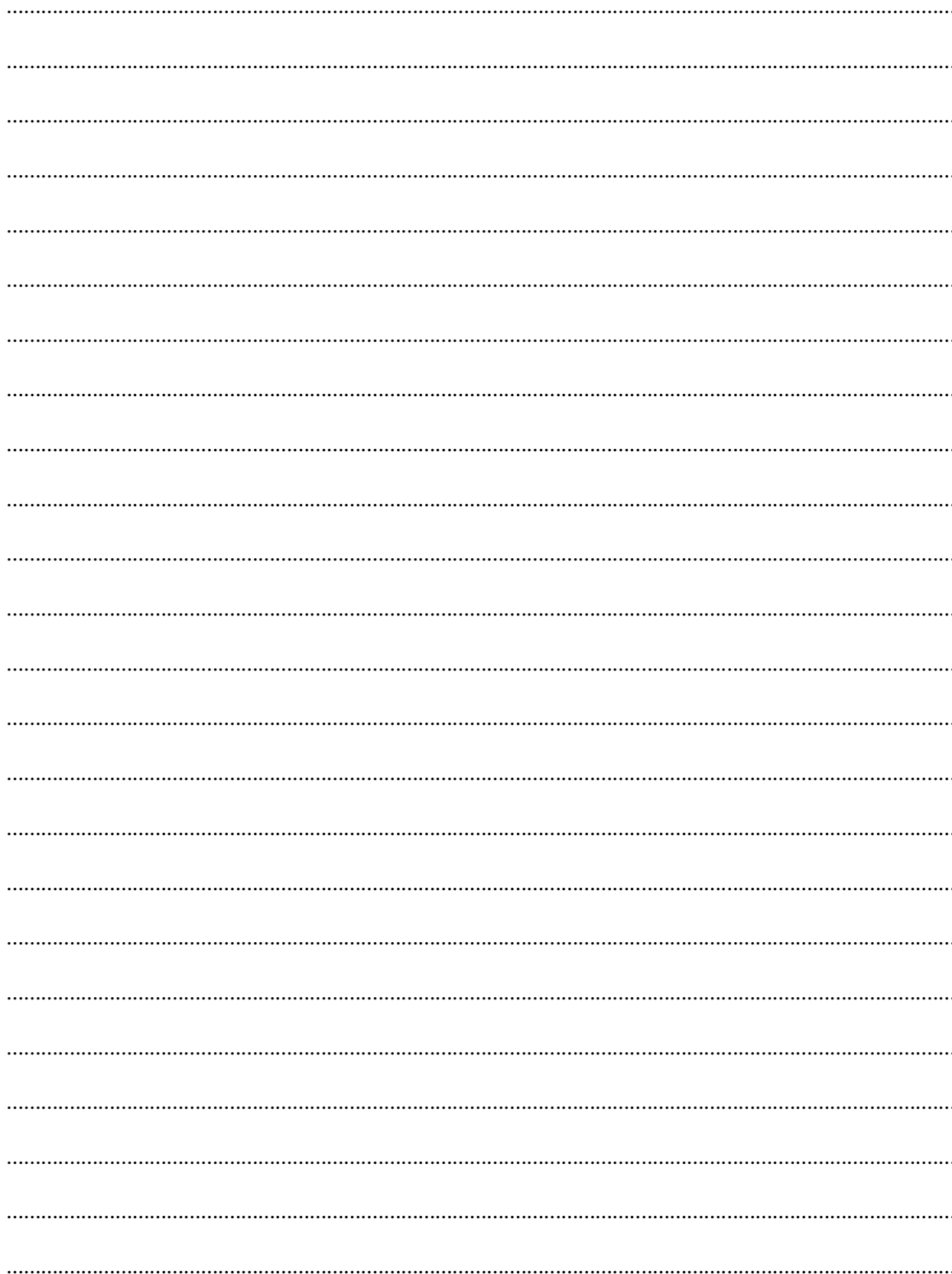
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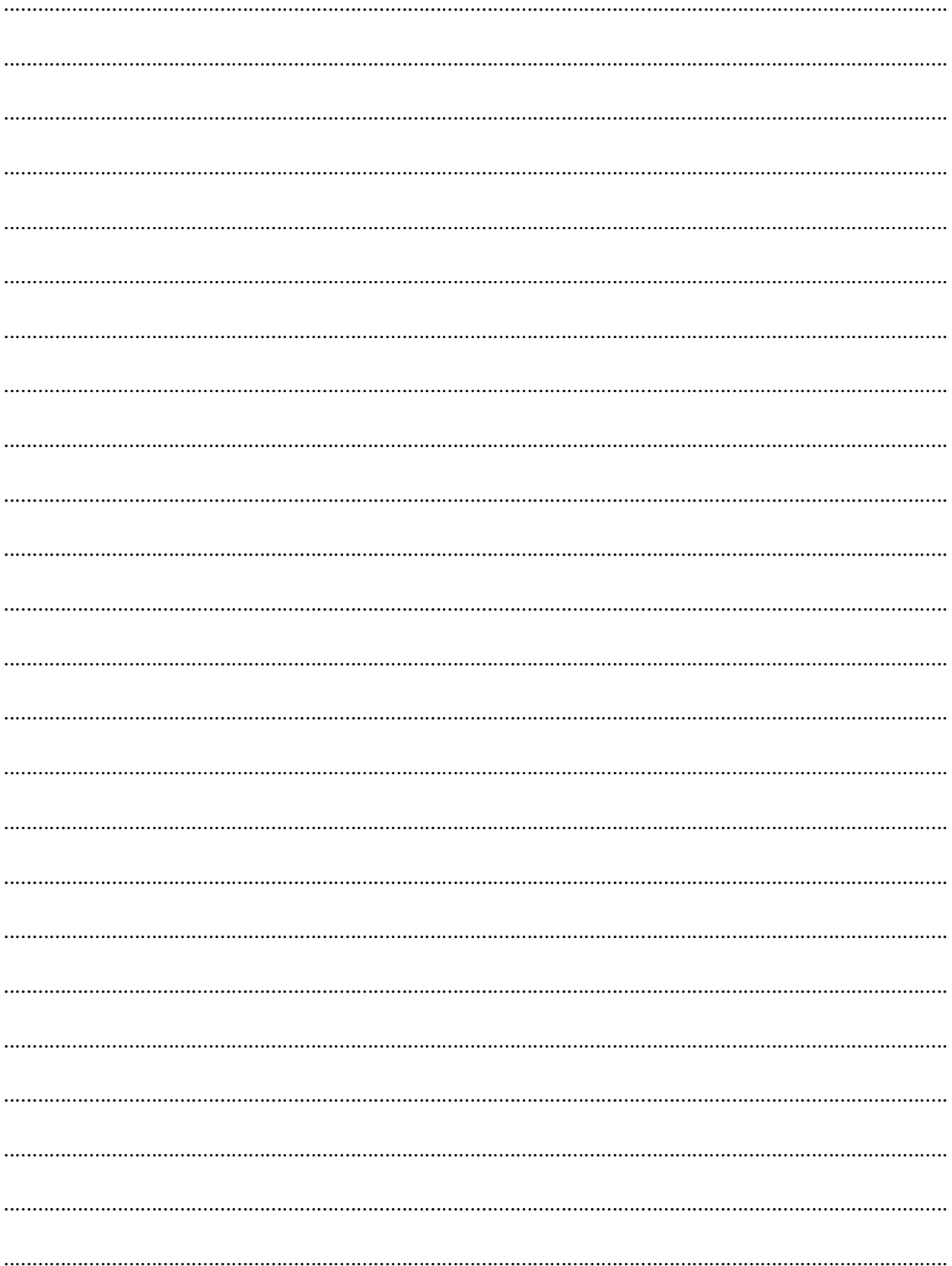














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