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## Novel Retaining Glycoside Hydrolases

### Potential candidates for transglycosylation and hydrolysis

Gulshan Kazi, Zubaida

2020

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*Citation for published version (APA):*

Gulshan Kazi, Z. (2020). *Novel Retaining Glycoside Hydrolases: Potential candidates for transglycosylation and hydrolysis*. Division of Biotechnology, Lund University.

*Total number of authors:*

1

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# Novel Retaining Glycoside Hydrolases

Potential candidates for transglycosylation and hydrolysis

KAZI ZUBAIDA GULSHAN ARA | BIOTECHNOLOGY | LUND UNIVERSITY





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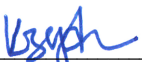
DOCTORAL DISSERTATION

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To be defended at Kemicentrum lecture hall B on 9<sup>th</sup> of September 2020 at  
14.15.

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<b>Organization</b> LUND UNIVERSITY Division of Biotechnology Box 124 SE-22100 Lund, Sweden  <b>Author(s)</b> Kazi Zubaida Gulshan Ara	<b>Document name</b> <b>Doctoral Dissertation</b>	
	<b>Date of issue</b> 2020-09-09  <b>Sponsoring organization</b> Amylomics (EU FP7), Formas, Thermofactories (via Formas), ProSeaFood (via Formas), Macro cascade (EU).	
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<b>Key words Glycoside</b> Glycoside hydrolase, transglycosylation, hydrolysis, alkyl glycosides, cyclodextrin glucanotransferases, <i>Rhodothermus marinus</i> , xylanases, beta-glycosidases		
Classification system and/or index terms (if any)		
Supplementary bibliographical information		<b>Language</b>
<b>ISRN</b> LUTKDH/TKBT-20/1177-SE		<b>ISBN</b> 978-91-7422-730-7 (print) 978-91-7422-731-4 (pdf)
Recipient's notes	<b>Number of pages</b> 196	Price
	Security classification	

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Faculty of Engineering  
Division of Biotechnology

ISBN 978-91-7422-730-7 (print)

ISBN 978-91-7422-731-4 (pdf)

ISRN LUTKDH/TKBT-20/1177-SE

Printed in Sweden by Media-Tryck, Lund University  
Lund 2020



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*To My shining little stars,*

*Eiliyah, Mahdi and Tihami*

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# Abstract

Our society is moving towards renewable resources, where biomass, rich in carbohydrates, is producing chemicals and fuel. However, there are several limitations when it comes to valorisation of the carbohydrates from renewable biomass. One major hurdle is the over-functional nature of carbohydrates, making them difficult to process by conventional chemistry. Carbohydrate active enzymes can help overcome this limitation by providing excellent tools to utilise renewable feedstocks, supplying competitive alternatives to the traditional chemical process.

The enzymatic toolbox is the green alternative when it comes to synthesis of glycoconjugates and to make the transition towards bioeconomy, use of these tools is an essential step. In nature, glycosylation is executed mostly by glycosyltransferases. However, they are not ideal for industrial applications due to their need to use expensive activated donors. Whereas, transglycosylases (classified under glycoside hydrolase families: GHs) do not need any activated donor, making them perfect candidates. The only limitation with them is that there are not many that have been characterised. Transglycosylases are classified in the same families as their hydrolysing counterparts, and are closely related in sequence and structure, making it difficult to select them based on sequence similarities. A typical exception from this is the cyclodextrin glucanotransferases (CGTases) which belongs to GH13.

This thesis investigates the transglycosylation activity of cyclodextrin glucanotransferases, for expanding the utilisation of transglycosylases. The focus of the work was on the elongation of the carbohydrate part of alkyl glycosides. A novel cyclodextrin glucanotransferase (*CspCGT13*) from *Carboxydocella* sp. was characterised and compared with available commercial enzymes to evaluate the applicability in alkyl glycoside modification. The novel enzyme showed significant coupling activity with  $\gamma$ -cyclodextrin as the donor, however it was not as efficient as the commercial CGTases. Later, the coupling activity was improved by protein engineering and bioinformatic analysis, making it a competitive candidate for alkyl glycosides modification.

The majority of the enzymes in the GH-families are hydrolases and are widely available. Using glycoside hydrolases in synthesis requires reduced hydrolytic activity. In this thesis, oligosaccharide synthesis was studied by using glycosides

hydrolases. Significantly reduced hydrolysis was achieved for an endo-xylanase from the thermophilic bacterium *Rhodothermus marinus* DSM 4252<sup>T</sup> through protein engineering. The enzyme variants displayed enhanced transglycosylation activity.

In addition, novel candidates from the enzymatic toolbox from another strain of this marine thermophilic bacterium were also investigated in this thesis, aiming to gain more insight into the hydrolytic mechanism used for saccharification processes. Six novel *exo*-hydrolases from a single GH family (GH3) originating from *R. marinus* DSM 4253 were characterised. The study showed these enzymes to have broad substrate specificities and activities at moderately high temperature. Also, more information was obtained regarding their structural features and genomic distributions, providing more knowledge to tailor the enzymes for industrial applications.

# Popular science

In the last decade, we have witnessed the impact of natural disasters on planet earth, ranging from increases in temperature during summer to devastating floods. This drastic change in the global climate has been anticipated due to the level of greenhouse gas emissions from fossil fuels. Unfortunately, not all countries of the world have put enough effort to limit the emission of greenhouse gas, making it a more significant challenge for the current decade. According to UN Emission Gap report 2019, the world will be warmer by 3.2 degrees Celsius by 2100 if we do not reduce the emissions.

Significant progress is being made in reducing the emissions from fossil fuels in the energy and transport sectors through the development of solar energy and alternative fuels. But fossil fuels are unfortunately not only used in the energy and transport sectors. Most of the chemicals produced in the world come from a handful of intermediates produced in fossil-based oil refineries, contributing to the greenhouse gas emission. To reduce the negative impact, renewable alternatives like biorefineries, where biomass is used as raw material, are on the rise. The processing of biomass is often done by chemical treatment, at high temperature and high pressure. However, chemical treatment is not environment-friendly as it generates a bulk amount of waste.

So, the question came to our mind, how can we make a greener process? If we look at nature, every living organism is efficiently extracting energy from the organic sources available on earth. For that, they use the indispensable toolbox called enzymes, which are proteins. Biomass mostly contains carbohydrates, so carbohydrate-active enzymes play an essential role in breaking down the biomass into its sugar ingredients and making them available for microorganisms. Additionally, these enzymes can also build up valuable products from the sugar ingredients. Due to their high potential enzymes are needed to make the transition to a sustainable economy possible.

This thesis was done at a research group that works on isolating new enzymes and developing processes to utilise raw materials from various biomass sources. The thesis focuses on understanding how glycoside hydrolases can catalyse break-down and build-up of compounds with sugars as building blocks. Moreover, how this information can be used to design enzymes for industrial applications, so that the society could become more sustainable. Two examples

of such an application of enzymes are the production of surfactants (for example alkyl glycosides) and oligosaccharides, where carbohydrates are used as raw material. Alkyl glycosides are made by attaching sugar molecules to an alcohol. They are widely used in our household products, like dishwashing detergents, laundry detergents, and also in pharmaceuticals and personal care products. Oligosaccharides, on the other hand, are used as food supplements and made by adding sugar molecules together. For example, xylooligosaccharides have potential health benefits by promoting good bacteria in our gut, and because of that they have widely been used in different food products.

The process that makes these products is known as glycosylation, where a sugar molecule is joined to another sugar molecule or to different kind of molecules. To do it with traditional chemistry is quite tricky, requires many steps and chemical catalysis. Thus, it is energy demanding and produces bulk chemical waste. With enzymes, it can be done in a single step without harsh chemicals. There is an enormous variety of carbohydrate-active enzymes among the glycoside hydrolases. Unfortunately, not all can be used for glycosylation, since some are instead specialists in breaking down carbohydrate bonds in the presence of water, a process known as hydrolysis. Glycoside hydrolases efficient at hydrolysis are also interesting because of their application in biofuel production.

During my thesis, I was able to tailor enzymes for the production of alkyl glycosides and xylooligosaccharides. In addition, a set of enzymes were also studied with high potential to catalyse deconstruction of carbohydrates for many applications such as biofuel production. These results help us to understand this valuable enzyme class of carbohydrate acting enzymes and its immense power to drive the bio-economy. It also brings us closer to the goal of being able to use a wide variety of glycoside hydrolases for biorefineries and reduce the negative impact of fossil-based industries.



# Populärvetenskaplig sammanfattning

Under det senaste decenniet har vi bevittnat effekterna av naturkatastrofer på jorden, allt från temperaturökningar under sommaren till förödande översvämningar. Denna drastiska förändring i det globala klimatet har kunnat förutspås som en följd av nivån på utsläpp av växthusgaser från fossila bränslen. Tyvärr har inte alla länder i världen gjort tillräckligt med ansträngningar för att begränsa utsläppen av växthusgaser, vilket gör det till en ännu viktigare utmaning för det nuvarande decenniet. Enligt FN:s årliga "Emissions gap report" från 2019 kommer världen att vara 3,2°C varmare år 2100 om vi inte minskar utsläppen.

Betydande framsteg görs när det gäller att minska utsläppen från fossila bränslen i energi- och transportsektorerna genom utveckling av solenergi och alternativa bränslen. Men, fossila bränslen används tyvärr även inom andra områden. De flesta kemikalier som produceras i världen kommer från en handfull petrokemikalier som produceras i oljeraffinaderier, vilket bidrar till utsläppen av växthusgaser. För att minska den negativa effekten är förnybara alternativ som bioraffinaderier, där biomassa av olika slag används som råmaterial, på frammarsch. Bearbetning av biomassa sker ofta genom kemisk behandling, vid hög temperatur och högt tryck, men kemisk behandling är dock inte alla gånger miljövänlig eftersom en stor mängd avfall ofta genereras.

Detta fick oss att ställa oss följande fråga, hur kan vi utveckla miljövänligare processer? Om vi ser på naturen, tar varje levande organism effektivt upp energi från tillgängliga organiska källor. För det använder organismerna den oundgängliga verktygslådan som kallas enzymer, dvs ett slags proteiner. Biomassa består till stor del av kolhydrater, och därför spelar kolhydrataktiva enzymer en viktig roll vid spjälkning av biomassa till olika sockerarter då de på så vis görs tillgängliga för mikroorganismer. Dessutom kan denna typ av enzymer också bygga upp värdefulla ämnen från dessa sockerarter. Tack vare enzyms stora potential bidrar de till att möjliggöra en övergång till en mer hållbar ekonomi.

Denna avhandling gjordes vid en forskargrupp som arbetar med att isolera nya enzymer och utveckla processer för att utnyttja råvaror från olika typer av biomassa. Avhandlingen fokuserar på förståelsen av hur glykosidhydrolaser kan katalysera nedbrytning och uppbyggnad av föreningar med socker som byggstenar. Används sedan denna information för att designa enzym för industriella applikationer, kan det bidra till att skapa ett mer hållbart samhälle.

Två exempel på sådan tillämpning av enzymer är produktion av surfaktanter (exempelvis alkylglykosider) och oligosackarider, där kolhydrater används som råmaterial. Alkylglykosider tillverkas genom att man sammanfogar sockermolekyler och en alkohol. Sådana produkter används ofta i våra hushållskemikalier, som diskmedel och tvättmedel, men även i läkemedel och produkter för personlig hygien. Oligosackarider å andra sidan används som kosttillskott och tillverkas genom att sockermolekyler adderas till varandara. Exempelvis är xylooligosackarider en kedja av sockermolekyler som kallas xylos. Xylooligosackarider har potentiella hälsofördelar genom att främja goda bakterier i tarmen. Därför använts de ofta som tillsatser i bröd och andra livsmedelsprodukter.

Processen där den här typen produkter bildas kallas glykosylering, och fungerar på så vis att en sockermolekyl kopplas ihop med en annan sockermolekyl eller med någon annan sorts molekyl. Att göra det med traditionell kemi är komplicerat och kräver många steg med hög temperatur och kemisk katalys. Således är det energikrävande och producerar mycket kemiskt avfall. Med enzymer kan det göras i ett enda steg utan starka kemikalier. Det finns en enorm variation av kolhydrataktiva enzymer bland glykosidhydrolaserna. Förutom enzymer involverade i glykosylering finns det inom denna grupp enzymer som istället används vid nedbrytning av kolhydratbindningar i närvaro av vatten, en process som kallas hydrolys. Glykosidhydrolaser effektiva vid hydrolys är intressanta på grund av deras tillämpning inom biobränsleproduktion.

Under arbetet med min avhandling har jag haft möjlighet att skraddarsy enzymer för produktion av alkylglykosider och xylooligosackarider. Därutöver studerades en uppsättning enzymer med stor potential att katalysera nedbrytning av kolhydrater till enkla sockerarter, som sedan kan användas som byggstenar i många tillämpningar. Sammantaget hjälper resultaten oss att förstå hur värdefulla dessa kolhydratmodifierande enzymer är, samt visar på deras goda förutsättningar att bidra till att driva arbetet med en utveckling mot ett mer hållbart samhälle framåt. Detta för oss också närmare målet att kunna använda en mängd olika glykosidhydrolaser för bioraffinaderier och därmed minska den negativa effekten från fossilbaserade industrier.

# List of Papers

This doctoral thesis is based on the following papers, referred to by their Roman numerals in the text and provided at the end of the booklet.

- I **Ara KZG\***, Lundemo P, Fridjonsson OH, Hreggvidsson GO, Adlercreutz P and Karlsson EN. (2015). A CGTase with high coupling activity using  $\gamma$ -cyclodextrin isolated from a novel strain clustering under the genus *Carboxydocella*. *Glycobiology*. **25**(5), 514-523.
- II Rather MY, **Ara KZG\***, Karlsson EN, and Adlercreutz P. (2015). Characterization of cyclodextrin glycosyltransferases (CGTases) and their application for synthesis of alkyl glycosides with oligomeric head group. *Process Biochemistry*. **50**(5), 722-728.
- III **Ara KZG\***, Linares-Pastén JA, Jönsson J, Vilorio M, Ulvenlund Stefan, Adlercreutz P and Karlsson EN. (2020). Engineering of cyclodextrin glycosyltransferase to improve synthesis of alkyl glycosides. (Manuscript).
- IV Teze D, Jiao Z, Wiemann M, **Ara KZG\***, Lupo R, Rønne ME, Carlström G, Duus JØ, Sanejouand YH, O'Donohue MJ, Karlsson EN, Faure R, Stålbrand H and Svensson B. (2020). Rational enzyme design without structural knowledge: a sequence-based approach for efficient generation of glycosylation catalysts. (Manuscript, preprints available in **ChemRxiv**).
- V **Ara KZG\***, Månberger A, Gabrisko M, Linares-Pastén JA, Jasilionis A, Fridjonsson OH, Hreggvidsson GO, Janecek S and Karlsson EN. (2020). *Scientific Reports*. **10**:1329.

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# My contribution to the papers

All work described in this thesis was performed under the supervision of Professor Eva Nordberg Karlsson and Professor Patrick Adlercreutz at the Division of Biotechnology.

- I** I planned and performed the characterisation of the novel CGTase in cooperation with Pontus Lundemo. I wrote and revised the manuscript together with all the co-authors.
- II** Rather MY. performed majority of the work and wrote the primary draft of the manuscript. I produced the enzyme *CspCGT13* and performed coupling activity with dodecyl-maltoside for the enzyme. I wrote the corresponding part of the manuscript and also took part during revision of the manuscript.
- III** I planned and performed most of the experimental work (excluding the mutational experiment). I wrote the manuscript, with help of the co-authors.
- IV** David Teze conceived the idea and proposed the mutations for *endo*-xylanase *RmXyn10A\_CM*. I performed all the experimental work for the *RmXyn10A\_CM* and wrote the corresponding part of the manuscript. I took active part in the discussion together with all the co-authors.
- V** I planned and performed the biochemical characterisation of all six enzymes. I wrote and revised the manuscript with help of the co-authors.



# 1 Introduction

In the last two decades concerns about global climate change and shortage of resources have brought the environmental and sustainability issues into focus. One of the key issues in this aspect is reducing the dependency on fossil feedstock and to do so we need to use renewable feedstock as raw material. Traditionally crude oil has been serving as the source for fuel and raw materials to vast numbers of chemicals and materials produced by petroleum industries. Thus, to make the economy sustainable, transition to bio-economy is necessary and for this we need competitive biorefineries, where renewable feedstock can be converted to bio-chemicals, materials and energy carriers.

The key constituent of available biomass on earth is carbohydrates, which makes them the most abundant renewable feedstock. To valorise this renewable feedstock, carbohydrate active enzymes play a crucial role in the bio-economy. They are essential for biomass degradation and conversion of the biomass polysaccharides into components that can be converted to bio-fuel (Fatih Demirbas 2009). However, they have also made huge impact in converting biomass into value-added products, like bio-chemicals and materials. Glycoside hydrolases are a diverse group of enzymes that act on carbohydrates. They are divided into different families by using sequence-based classification. To grasp the power of the versatility of these enzymes, we need to look back at the nature of the carbohydrates. Carbohydrates are made of sugar units which can be joined together in a multitude of ways. For example the number of possible isomers for hexa-saccharides are more than  $10^{12}$  (Laine 1994). Thus, characterisation of new enzymes with novel activity is very demanding. Moreover, there is also a great need for making cost effective processes for biorefineries to compete with fossil-based processes.

Glycoside hydrolases have been investigated widely for biomass saccharification and for their use in synthesis processes, with specifically significant advancement achieved in the bio-fuel area. In bio-chemical synthesis processes, addition of a sugar unit onto a molecule of interest, also known as glycosylation, catalysed by enzymes can provide significant improvement of the physio-chemical properties, such as stability (Jun, Bae et al. 2001) and solubility (Slámová, Kapešová et al. 2018) in aqueous medium. This principle can be used in synthesis of various value-added products, ranging from bio-based surfactants to functional oligosaccharides synthesis. Considering the diverse applications in biorefineries,

glycoside hydrolases with enhanced and tailored catalytic functions are highly desirable. The work presented in this thesis has been focused on two different aspects; understanding the structure-function relationship of novel retaining glycoside hydrolases from thermophilic organisms and improving their functionality for modification of alkyl glycosides and synthesis of xylooligosaccharides (XOS).

## 1.1 Scope of the thesis

The thesis is founded on five manuscripts, of which three are published and two are in the form of manuscripts.

**Paper I-III**, deal with transglycosylation by cyclodextrin glucanotransferases, aiming towards producing alkyl glycosides with varied length of the carbohydrate chain. In **Paper I**, a novel cyclodextrin glucanotransferase (CGTase) was studied and compared with other commercial CGTases. **Paper II** describes the potential application of the novel CGTase in alkyl glycoside synthesis and a comprehensive study of the reaction products in comparison with other CGTases. In **Paper III**, the novel CGTase was subjected to protein engineering to improve the production of alkyl glycosides.

**Paper IV** describes a novel protein engineering approach to overcome the hydrolytic activity of an *endo*-xylanase and enhance the transglycosylation activity. This approach was investigated for xylooligosaccharides synthesis.

In **Paper V**, the focus was on hydrolysis instead of transglycosylation. It describes the characterisation of six *exo*- $\beta$ -glycosidases from a thermophilic bacterium with a fundamental approach to understand their catalytic mechanism and substrate specificities which will be beneficial for their utilisation in biorefinery applications.

## 2 Glycoside Hydrolases

Glycoside hydrolases (GHs) are a major class of enzymes widespread in our surrounding nature. They hydrolyse the glycosidic bonds present in carbohydrate polymers (Sinnott 1990). Glycoside hydrolases have a wide range of activities, which are dependent on the type of glycosidic linkage of substrates they act on. Their diversity is also enriched by the surroundings of the host organisms, such as glycoside hydrolases from thermophilic bacteria are more robust compared to others.

### 2.1 Classification

Glycoside hydrolases can be classified following various criteria. The most implemented one is the classification system attributing GHs into families based on sequence comparison (Henrissat 1991). At present there are 165 GH family listed in the Carbohydrate-Active enZymes database (CAZy) ([www.cazy.org](http://www.cazy.org)). However, based on the overall enzyme folding, GH families are further divided into different clusters known as clans (Henrissat and Bairoch 1996). Despite their similarity in sequences and structures, a few families harbour enzymes with different substrate specificity and those are further classified into subfamilies. Such an example is GH family 13 which contains thousands of sequences with a large number of substrate specificities, and is divided into 42 subfamilies (Stam, Danchin et al. 2006) ([www.cazy.org](http://www.cazy.org)).

Glycoside hydrolases can be categorised as *exo*- or *endo*-acting, depending on their ability to cleave a substrate at the end of the chain or in the middle of it respectively (Davies and Henrissat 1995).

In addition to the presented classifications, GHs are also classified by their reaction mechanisms and the two most common mechanisms performed by them are retaining and inverting. The principal behind this nomenclature is whether the anomeric configuration of the hydrolytic product is fully retained or inverted during the course of the reaction (Koshland Jr. 1953, Gebler, Gilkes et al. 1992).

## 2.2 Glycoside hydrolases investigated in this thesis

This thesis is comprised of a discussion on retaining GHs from three different families, which includes GH3, GH10 and GH13. The nomenclature of the enzymes studied here follow the convention proposed by Henrissat et al. (Henrissat, Teeri et al. 1998). The first letters in italic, indicates the host organism, followed by three letters identifying the substrate it acts on, the respective GH family number and finally a capital letter, to separate multiple occurrences of genes from the same microorganism, encoding enzymes from the same GH-family. Enzymes that will be discussed throughout this thesis are listed below in Table 1. In addition to this, three commercial cyclodextrin glucanotransferases (CGTases) will also be addressed (**Paper I-II**). All the further discussions on overall structural features and mechanisms will be focused on retaining glycoside hydrolases.

**Table 1:** List of enzymes studied in the thesis

Name of enzymes	GH family
<i>endo</i> -acting glycoside hydrolases	
CspCGT13 ( <b>Paper I-III</b> )	GH 13
RmXyn10A_Catalytic Module ( <b>Paper IV</b> )	GH 10
<i>exo</i> -acting glycoside hydrolases	
RmBgl3A, B and C ( <b>Paper V</b> )	GH 3
RmXyl3A and B ( <b>Paper V</b> )	GH 3
RmNg3 ( <b>Paper V</b> )	GH 3

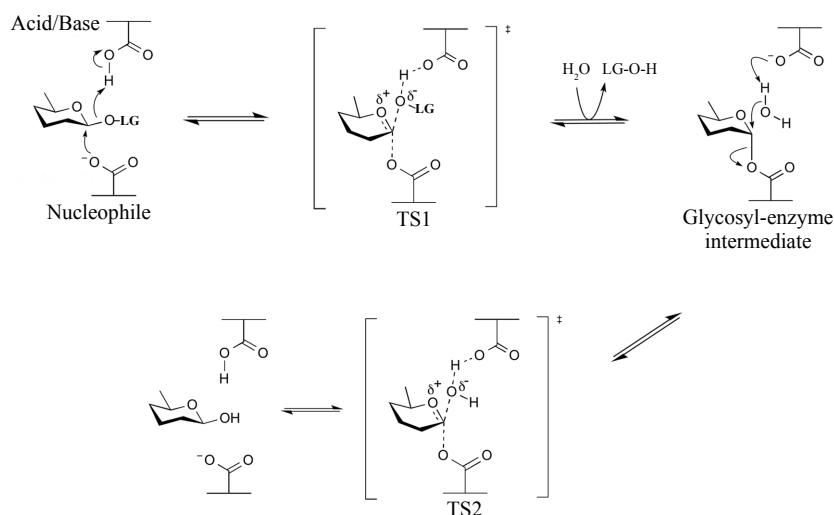
## 2.3 Folding and structural modularity

Structural information is necessary to understand the evolutionary relationships between vast groups of enzymes such as GH families. Furthermore, it can also provide a detailed comprehension on folds, structural modularity and catalytic functions (Henrissat, Sulzenbacher et al. 2008). As mentioned earlier, GH families are distributed under different clans based on the overall fold, which represents the arrangement of the secondary structure and are highly conserved. There are seven different folds described for glycoside hydrolases and the TIM barrel fold occurs most commonly. The over representation of TIM barrel can be explained by their versatility achieved through evolution (Henrissat, Callebaut et al. 1995).

Conversely, the structural modularity of glycoside hydrolases varies more than the overall fold. Additionally to the catalytic module (often called domain), GHs

can also have non-catalytic domains (auxiliary domains), which are often binding modules appended (Cantarel, Coutinho et al. 2008). Very well-studied examples are the Carbohydrate-binding modules (CBMs), which promote the recognition and binding of carbohydrates (Boraston, Bolam et al. 2004). However, in many cases, the functions of the non-catalytic modules are not well known.

Structures of enzymes in GH3, 10 and 13, in most cases are multi-domain proteins. In GH3, all enzymes have domains containing both the  $(\alpha/\beta)_6$  fold and the classical  $(\alpha/\beta)_8$  TIM barrel fold, but these enzymes are not classified under any clan yet (Pozzo, Pasten et al. 2010). In contrast, GH10 enzymes belong to clan GH-A and have  $(\alpha/\beta)_8$  TIM barrel fold. Similarly, all enzymes in GH13 belong to the clan GH-H, where the catalytic domain has TIM barrel fold but the C-terminal domain contains a  $\beta$ -sandwich fold (Janecek, Svensson et al. 1997).



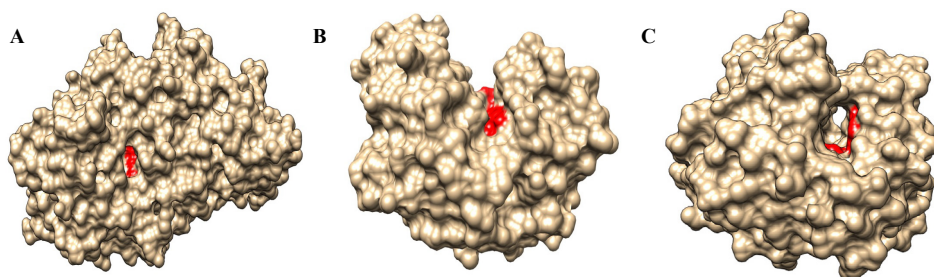
**Figure 1:** The Classical retaining mechanism performed by  $\beta$ -glycosidases (Consortium 2017). TS1 refers to the first transition state and TS2 to the second transition state. LG = leaving group.

## 2.4 Catalytic residues and mechanism

The catalytic activity (hydrolysis or transglycosylation) by retaining glycoside hydrolases occurs in the active site of the catalytic domain, which is built up of carboxylic amino acids acting as acid/base and nucleophile (Rye and Withers 2000). However, some retaining GHs that catalyse substrates containing an *N*-acetyl (acetamido) or *N*-glycolyl, use acetamido group as an intramolecular

nucleophile (Terwisscha van Scheltinga, Armand et al. 1995, Mark, Vocadlo et al. 2001).

In the retaining mechanism (also known as Classical Koshland retaining mechanism) the catalysis happens in two steps (Fig. 1). Firstly, a nucleophile residue attacks on the anomeric carbon and breaks the glycosidic bond with help of the catalytic acid, which donates a hydrogen atom, enabling the release of the aglycone product. Subsequently, a covalent glycosyl enzyme intermediate of opposite anomeric configuration is formed with the glycone product. In the second step, a water molecule in case of hydrolysis (or an acceptor molecule in case of transglycosylation) deprotonated by the catalytic acid attacks the covalently bound anomeric carbon and releases the glycone or the second product (Koshland Jr. 1953).



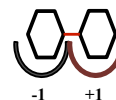
**Figure 2:** Representation of the active site topology of glycoside hydrolases. (A) pocket illustrated by the  $\beta$ -glucosidase from *Hordeum vulgare* subsp. *vulgare* (PDB:1EX1), (B) cleft illustrated by the xylanase from *Cellulomonas fimi* (PDB:1EXP). (C) tunnel illustrated by the catalytic domain of the cellobiohydrolase from *Coprinopsis cinerea* (PDB:3VOG). The substrate interacting residues are coloured in red.

## 2.5 Active site topology and nomenclature

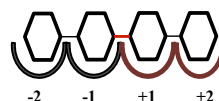
Although GHs has structural diversity, the overall active site topology does not differ significantly. It can be divided into three groups namely, pocket, cleft and tunnel topology (Fig. 2A-C). The first one, pocket topology (Fig. 2A) is commonly found in *exo*-acting enzymes and for enzymes active on short substrates, whereas the cleft and tunnel topologies (Fig. 2B-C) are present in *endo*-acting enzymes active on polymeric substrates (Davies and Henrissat 1995). Tunnel topology is derived from the previous one when long loops covered parts of the cleft and it allows holding the substrate while releasing the product. The CGTases

(**Paper I-III**) and the *endo*-xylanase (**Paper IV**) have cleft topology (Fig. 2B) while *exo*-hydrolases from GH 3 (**Paper V**) have pocket topology (Fig. 2A).

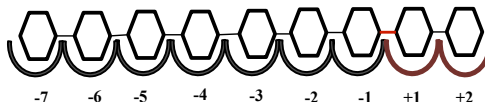
*RmBgl3A,B,C/RmXyl3A,B/RmNag3*



*RmXyn10A\_CM*



*CspCGT13*



**Figure 3:** Schematic overview of the substrate binding sites for *exo* and *endo*-acting glycoside hydrolases. The binding subsites are labelled from -7 to +2 where the point of cleavage is between subsite -1 and +1, the bond is indicated with red colour. *CspCGT13* and *RmXyn10A\_CM* are representing the *endo*-acting GHs while *RmBgl3A,B,C* ( $\beta$ -glucosidases); *RmXyl3A* and *B* ( $\beta$ -xylosidases) and *RmNag3* ( $\beta$ -N-acetyl-glucosaminidase) represent *exo*-acting GHs.

The active sites of GHs contain several sub-sites where the saccharide units of the carbohydrate substrates can bind. They are labelled from -n to +n, where n is an integer. The numbering starts from the bond that will be cleaved and continues as -n towards non-reducing ends and +n towards reducing end of the substrate (Davies, Wilson et al. 1997). The minus sub-sites are also referred to as the glycone subsites and the positive as aglycone subsites (Fig. 3). The influential role of active site architecture in substrate specificity is explored for *exo*- $\beta$ -glycosidases from family GH3 in chapter 6 and **Paper V**.

# 3 Glycosylation by glycoside hydrolases

Synthesis of carbohydrate-based compounds has become very important for both understanding the fundamentals in glycosciences and for the conversion of them into value added products. This conversion can be achieved by glycosylation that involves adding a glycosyl group (donor) to the functional group of another molecule (acceptor). There are two routes to do the glycosylation process, either using conventional carbohydrate chemistry or enzymatic synthesis. However, the enzymatic synthesis has become more desirable since it requires a single step while the chemical synthesis involves multiple steps (protection, activation, glycosylation and deactivation) (Koenigs and Knorr 1901). Also, the end product is anomerically pure in enzymatic glycosylation, which is a major limitation for the chemical process.

Although occurring in nature, the glycosylation process is mainly executed by a group of enzymes called glycosyltransferases (GTs) that uses both nucleotide and non-nucleotide sugars as donor substrates (Lairson, Henrissat et al. 2008, Breton, Fournel-Gigleux et al. 2012). GTs that utilise nucleotide donors are called Leloir GTs whereas non-nucleotide donors are used by non-Leloir GTs. The use of Leloir GTs in industrial processes is severely limited due to the price and low availability of the nucleotide substrates (Field 2011, Gantt, Peltier-Pain et al. 2011). In general, the heterologous expression of glycosyltransferases is also challenging which make them less attractive for synthesis applications (Bissaro, Monsan et al. 2015). Considering the drawbacks of GTs, other carbohydrate active enzymes are used more frequently for glycosylation, such as transglycosylases classified under glycoside hydrolase families.

## 3.1 Transglycosylases (TGs)

Transglycosylases (TGs) are a group of retaining GHs that predominantly catalyse transglycosylation, specifically the intra- or inter-molecular substitution at the anomeric position of a glycoside. They are classified as glycoside hydrolases under different GH-families. These enzymes are ideal for performing



glycosylation via transglycosylation. There are few well studied TGs available in the databases, and their specificity spectrum is narrower compared to other GHs. Cyclodextrin glucanotransferase is classified as TG and belongs to GH family 13. **Paper I-III** are focused on the functionality of this type of TG and discussed in chapter 5.

## 3.2 Glycoside hydrolases (GHs)

Glycoside hydrolases have become more popular as an alternative to TGs due to their abundance and broad substrate specificities. Donor substrates used by GHs are often cheap and come from renewable resources. The retaining glycoside hydrolases can also catalyse the formation of glycosidic bonds (but to a lesser extent compared to the hydrolysis reaction and under certain conditions), therefore suppressing the hydrolytic activity could substantially improve the synthesis property. This can be achieved through reverse hydrolysis by shifting the thermodynamic equilibrium of the reaction where transglycosylation is forced against hydrolysis (Cote and Tao 1990). Reverse hydrolysis occurs through condensation of donor and acceptor, resulting in corresponding glycoside and water as leaving group. This reaction is under thermodynamic control and the yield of glycosylated product is determined by the equilibrium constant. Increasing substrate concentration and reducing the amount of water in the reaction system can shift the equilibrium. However, the reduced water concentration has effect on the enzyme activity because glycosidases are not very active at low amounts of water (Ljunger, Adlercreutz et al. 1994).

Another approach is transglycosylation which is controlled by kinetics. In this case, GHs can use an external acceptor other than water for the second step of the deglycosylation, resulting in the formation of a new glycan (Van Rantwijk, Woudenberg-van Oosterom et al. 1999). In the presence of water and another acceptor molecule both hydrolysis and transglycosylation take place and the level of glycosylation is often defined by the ratio between hydrolysis and transglycosylation (H/T). The GHs also suffer from other drawbacks, such as secondary hydrolysis when the transglycosylation products are further hydrolysed, resulting in lower yields. Another potential drawback is poor regioselectivity especially when the acceptor molecule contains multiple hydroxyl groups leading to a mixture of products. Utilisation of transglycosylation in synthesis using different acceptor molecules is mentioned in the next chapter. While, the mechanism is discussed in more detail in chapter 6, where an *endo*-xylanase from GH10 has been studied for synthesis (**Paper IV**).

### 3.3 Strategies to improve glycosylation in GHs

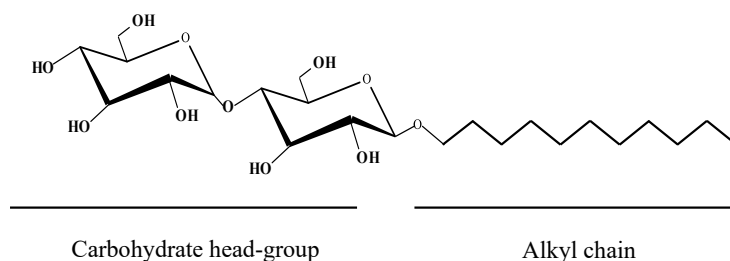
In general, the scientific community has for a long time been working on the development of different tools to improve enzymatic glycosylation. A considerable amount of research has been dedicated towards the understanding of the molecular aspects of the transglycosylation mechanism as well as enhancing the toolbox for making efficient biocatalysts.

The main focus of most of the studies has been on finding the key molecular elements that direct the selectivity between water and other acceptor substrates in GHs. Additionally, protein engineering has also been used to reduce the hydrolytic activity and turning GHs into pseudo-TG for different applications. For example, two popular approaches that have been used are directed evolution and rational design aimed at getting active site residues to influence the interactions in glycone and aglycone regions, as well as affecting the binding of water molecules (Kuriki, Kaneko et al. 1996, Aghajari, Roth et al. 2002). Different approaches to improve the transglycosylation activity are further discussed in chapter 4 (transglycosylases, TG), 5 ( $\beta$ -retaining GHs) and in Paper III-IV.

Another important progress in this area was the development of the glycosynthase concept, which was proposed initially by Withers and then by Planas in 1998 (Mackenzie, Wang et al. 1998, Malet and Planas 1998). Glycosynthases are glycosidases where the catalytic nucleophile residue is replaced by a smaller non-charged residue resulting in inactive variants with reduced or no hydrolytic activity. By using an activated donor such as glycosyl fluoride substrates the inactivated variants can perform glycosylation. Glycosynthase approach has been extensively studied and is summarised by a few outstanding reviews (Vasella, Davies et al. 2002, Vuong and Wilson 2010, Cobucci-Ponzano and Moracci 2012). Although it has been a remarkable step towards efficient glycosylation, glycosynthases are dependent on expensive activated glycosyl donors.

## 4 Application of transglycosylation in synthesis

Synthesis of carbohydrate-based compounds is crucial for the transition to a biobased economy. There is a considerable interest from food, pharmaceutical and cosmetic industries for the synthesis of defined glycoconjugates, such as oligosaccharides, surfactants, glycolipids and so on. In all these cases the transglycosylation strategy has been applied successfully (Usui, Matsui et al. 1990, Wang and Huang 2009, Leemhuis, Kelly et al. 2010, Hoffmann, Grey et al. 2020). Although this area is vast and diverse, the following sections will focus on advances made in the field of alkyl glycoside and oligosaccharides synthesis, using transglycosylation catalysed by retaining GHs.



**Figure 4:** Illustration of dodecyl- $\beta$ -maltoside, a non-ionic surfactant.

### 4.1 Alkyl glycosides synthesis and modification

Alkyl glycosides are nonionic biobased surfactants consisting of a hydrophilic carbohydrate group as head and a hydrophobic alkyl chain as tail, such as dodecyl- $\beta$ -maltoside (Fig. 4). They can have varied numbers of carbohydrate groups as well as varied lengths of the alkyl chain, making them diverse in nature. Large-scale production of alkyl glycosides involves the Fischer glycosylation process, which was first described by Emil Fischer in the 19th century (Fischer 1893). However, the chemo-, regio- and stereoselectivity is poor in chemical

synthesis and this can be improved by enzymatic synthesis. Enzymatic synthesis of alkyl glycosides can be achieved via two routes, reverse hydrolysis or through transglycosylation. Only modifications obtained via transglycosylation are presented below.

#### 4.1.1 Synthesis

Most of the reported synthesis of alkyl glycosides were catalysed by retaining *exo*- $\beta$ -glycosides (Fischer, Bromann et al. 1995, Lundemo, Karlsson et al. 2017). A few *endo*-GHs have also been reported for production of alkyl xylosides and mannosides. The alkyl glycoside synthesis using transglycosylation is highly dependent on the activated substrate as donor. The advantage of using activated donors like aryl glycosides, is high reaction rates due to the good leaving groups (von Rybinski and Hill 1998, Van Rantwijk, Woudenberg-van Oosterom et al. 1999, Andersson and Adlercreutz 2001). Moreover, selectivity of the alcohol group can also be modulated by the type of enzyme used as catalyst. This approach is more applicable for shorter alcohols (Benešová, Lipovová et al. 2009).

#### 4.1.2 Modification

The length of the carbohydrate head-group of alkyl glycosides has significant effect on the surfactant properties. For example, the Kraft temperature, defined as the critical temperature at which the surfactant is highly soluble. Below these temperature surfactants form crystalline structures and above they form micelles (Hayes and Smith 2019). To increase the solubility of alkyl glycosides it is important to increase the number of carbohydrate head groups which will also lower the Kraft temperature (Ulvenlund, Andersson et al. 2016). Moreover, alkyl glycosides with increased length of hydrophobic (alkyl) and hydrophilic (glycoside) chain have lower cellular toxicity (Ekelund, Östh et al. 2005). Thus, there is a substantial interest in the modification of alkyl glycosides, specifically for the elongation of the glycoside head group.

The elongation of the glycoside head group can be achieved through transglycosylation by enzymes classified as GHs, more specifically the CGTases. The synthesis is obtained through intermolecular transglycosylations catalysed by CGTases. The elongation process mostly produced alkyl glycosides with varied range of degree of polymerisations in head groups (Yoon and Robyt 2006), until, Svensson and co-workers improved the process significantly towards making alkyl glycosides with defined length of oligomeric head groups. They were able to add 6 glucose units to dodecyl-maltoside (Fig.4) by using CGTases in a single step (Svensson, Ulvenlund et al. 2009). The reaction mechanisms of CGTases involved in the synthesis of well-defined alkyl glycosides are further

discussed in chapter 5 and **Paper I** and **III**. In addition, applications of different CGTases are studied in **Paper I-II**.

### 4.1.3 Applications of alkyl glycosides

Alkyl glycosides are commonly used in household cleaning agents like detergents. They have effective cleaning properties within a wide pH range that make them highly applicable in all-purpose cleaning solutions with slightly acidic pH as well as in alkaline dishwasher detergents (von Rybinski and Hill 1998). Alkyl glycosides are mild and have foaming boosting (for anionic surfactants) properties, which make them very useful for formulations of the personal care products. For example, shampoo with alkyl glycosides showed lower reduction of the tensile strength of hair than other surfactants (Busch, Hensen et al. 1994). In pharmaceutical industries alkyl glycosides are applied in drug formulation to increase the bioavailability of the therapeutic agent (Maggio 2012). They are also used in antimicrobial product formulations due to their potential anti-microbial activity (Matsumura, Imai et al. 1990). Additionally, they are widely used for the extraction and crystallisation of membrane proteins (le Maire, Champeil et al. 2000).

## 4.2 Oligosaccharides synthesis

In recent years the research area in functional foods took a major turn due to the high demand among consumers. Oligosaccharides with high nutritional value gained a huge interest, since they have been widely used as food additives, dietary supplements and prebiotics (Prapulla, Subhaprada et al. 2000, Oku and Nakamura 2002, Broekaert, Courtin et al. 2011). Also other functional oligosaccharides have become more popular, like the lactose-based human milk oligosaccharides which have a great niche in infant food formulations (Bode 2012, Triantis, Bode et al. 2018). This shift towards health promoting effects of oligosaccharides leads to an increased need for consistent large-scale production. Generally, most large-scale productions of oligosaccharides come from biomass extracted via chemo-enzymatic processes, e.g. maltodextrins produced from starch that can be isolated from different crops. Thus the process varies a lot based on the type of biomass and sometimes requires chemicals that are harmful for the environment (Prapulla, Subhaprada et al. 2000). Also, oligosaccharides obtained from this process need further purification before being used in food applications. To overcome these limitations, enzymatic synthesis is used (Patel and Goyal 2011). Both GTs and GHs have been used in the synthesis of defined-length oligosaccharides (Crout and Vic 1998, Weijers, Franssen et al. 2008). Production of oligosaccharides by GHs using transglycosylation is well summarised in the review article by Manas

et al. that show the potential usage of the transglycosylation reaction in this area (Abdul Manas, Md. Illias et al. 2018).

#### **4.2.1 Xylooligosaccharides (XOS)**

Xylooligosaccharides have various applications in food, feed and pharmaceutical industries (Vázquez, Alonso et al. 2000). The non-digestibility property of XOS has made them more interesting as prebiotics and in recent years numerous studies have been conducted on their salutary effect on gut microbiota (Falck, Precha-Atsawan et al. 2013, Patel, Falck et al. 2013, Ho, Kosik et al. 2018). The production of XOS from lignocellulosic biomass involves physical, chemical and/or enzymatic hydrolysis (Vázquez, Alonso et al. 2000). Enzymes from thermophilic origin have shown to be beneficial for producing XOS from biomass (A Linares-Pasten, Aronsson et al. 2018). Utilisation of the transglycosylation reactivity by an *endo*-xylanase for XOS synthesis is demonstrated in chapter 6 and **Paper IV**.

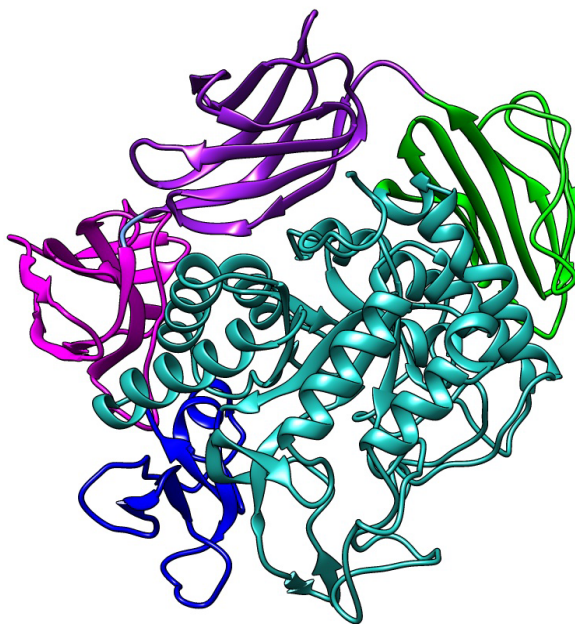
## 5 Transglycosylation by cyclodextrin glucanotransferases

GH family 13 is a family that comprise a very large number of enzymes acting on a wide range of substrates containing the  $\alpha$ -glucoside linkages, and it has subsequently been divided into sub-families. Cyclodextrin glucanotransferases (CGTases) belong to GH13 sub-family 2 and to clan GH-H. The most defining property of CGTases is the capability to produce cyclodextrins from starch. Cyclodextrins are cyclic oligosaccharides with 6, 7 or 8 glucose units known as  $\alpha$ ,  $\beta$  or  $\gamma$ -cyclodextrins respectively. They have a long history of being applied in food, pharmaceuticals and cosmetics industries due to the ability to form inclusion complexes with hydrophobic molecules (Del Valle 2004).

Nevertheless, CGTases are classified under the only subfamily in GH13 that has been characterised as containing transglycosylases (TGs). Unlike other TGs, CGTases have the ability to perform intra-molecular transglycosylation (cyclization) as well as inter-molecular transglycosylation using both linear (disproportionation) and cyclic (coupling) substrates. This uniqueness of CGTases makes them an ideal candidate to be applied in synthesis applications and hence fit to the scope of this thesis. The key aspects that influence the ratio between the described transglycosylation reactions are discussed in this chapter.

### 5.1 Comparison between $\alpha$ -amylases and CGTases

Enzymes from GH13 are multi-domain and hence the overall sequence similarities are relatively low within the family. The number of domains may vary within GH13, but the catalytic residues are highly conserved. The CGTases and their hydrolytic counterparts  $\alpha$ -amylases, have similar structural features (Nakajima, Imanaka et al. 1986, Svensson 1994, van der Veen, Uitdehaag et al. 2000). Previously it has been proposed that CGTases have evolved from the hydrolytic  $\alpha$ -amylases and work in synergy to facilitate the saccharification of starch (del-Rio, Morett et al. 1997).



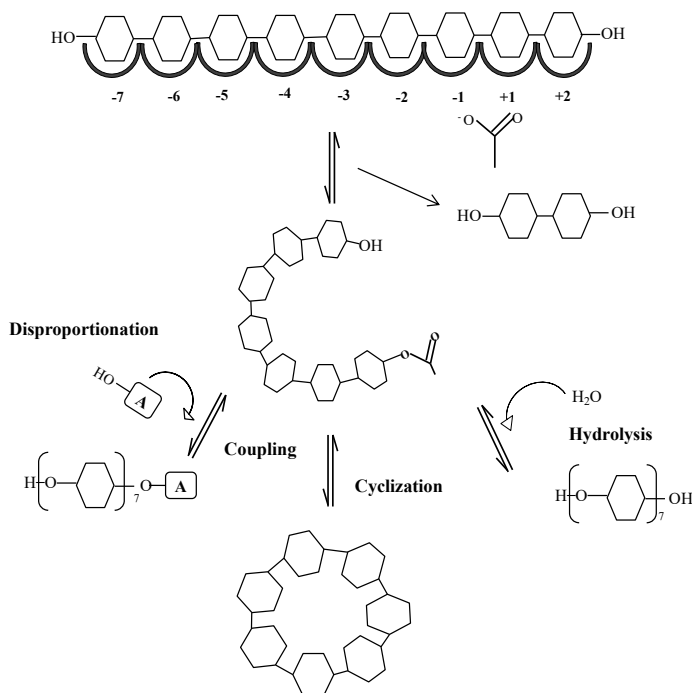
**Figure 5:** Structural representation of the cyclodextrin glucanotransferase from *Bacillus circulans* 251 (PDB:1CDG). Domain A displayed in light sea green, domain B in blue, domain C in green, domain D in purple and domain E in magenta.

The  $\alpha$ -amylases consist of three domains, A, B and C, while in CGTases, two additional domains: domain D and E are also present (Fig.5). Interestingly, domain E which is a starch binding domain (SBD) is not exclusive to GH13 but also found in other starch-degrading enzymes such as glucoamylase (Svensson, Larsen et al. 1986). Indeed, the additional domains bound to the C-terminal end of CGTases are a major difference to  $\alpha$ -amylases and contribute to the reaction specificity of these enzymes. Moreover, the number of subsites involved in the interactions with substrates also varies between these two enzyme types ( $\alpha$ -amylases and CGTases). For example the structure analysis of CGTases revealed subsites extending from -7 to +3 while in Porcine pancreatic  $\alpha$ -amylase (PPA) only five subsites (-3 to +2) are described (Ajandouz and Marchis-Mouren 1995, van der Veen, Uitdehaag et al. 2000). Another key difference observed is an extension of a loop in amylases that hinder substrate binding in -3/-4 subsites, discussed later in section 5.3.1 (Beier, Svendsen et al. 2000). It is also important to mention, that the residue at position 195 (numbering according to the CGTase from *Bacillus circulans* 251) is either a tyrosine or phenylalanine in CGTases where  $\alpha$ -amylases have an amino acid with short side-chain (Penninga, Strokopytov et al. 1995).



## 5.2 Reaction mechanism

The CGTases act exclusively on the  $\alpha$ -(1,4)-bond and are endo-acting where they use the double-displacement mechanism (for hydrolysis) like all other retaining GHs. They also have an extra catalytic residue conserved (Asp) for transition state stabilisation (Brzozowski and Davies 1997). The catalytic triad and four additional residues are conserved in the -1 subsite in both CGTases and  $\alpha$ -amylases. These residues are involved in substrate binding and distortion in CGTases (Uitdehaag, Mosi et al. 1999).



**Figure 6:** Schematic representation of reactions catalysed by CGTases. Glucose units are represented as hexagon shapes.

CGTases catalyse four different reactions and the catalysis starts by cleaving the  $\alpha$ -1,4-glycosidic bond of the substrate between the -1 and +1 subsites resulting in formation of a  $\beta$ -glycosyl intermediate. After the dissociation of the first product, binding of an acceptor molecule is allowed in the aglycone subsite which can be the non-reducing end of the covalently linked glycone making the cyclic rings or water in hydrolysis. The glycosyl-intermediate can also be transferred to other

acceptor molecules with a hydroxyl group resulting in disproportionation and coupling (Fig.6).

Remarkably, the two transglycosylation reactions catalysed by CGTases follow different kinetic mechanisms. The disproportionation reaction has been shown to follow the ping-pong mechanism specific for two-substrate reactions, while the coupling reactions proceed by a ternary complex mechanism where both donor and acceptor bind to the active site simultaneously in a random order (van der Veen, van Alebeek et al. 2000). Although, binding of a cyclodextrin as a donor in the active site would cause hindrance for other molecules to reach the acceptor subsites. The acceptor could, however, be partially bound to the +2/+3 subsites. The study by van der Veen and co-workers proposed that the acceptor bound to the +2 subsite subsequently moves to the +1 subsite after the formation of a covalent enzyme-intermediate. This shifting of the acceptor molecule is more favoured than linearization of the covalently linked cyclodextrins thus resulting in a ternary complex (van der Veen, van Alebeek et al. 2000).

## 5.3 Altering the reaction specificities in CGTases

As CGTases perform more than one type of transglycosylation reaction, alteration of the ratio between transglycosylation and hydrolysis (T/H) is more complicated for these enzymes than for other retaining GHs. To affect the reaction specificities, both glycone (donor) and aglycone (acceptor) subsites in CGTases have been subjected to several mutagenesis approaches to obtain desired reaction products.

### 5.3.1 Interplay between cyclization and hydrolytic activity

As mentioned above, the GH13 CGTases can catalyse cyclization (intra-molecular transglycosylation) through the transfer of the covalently bound donor molecule on to its own 4-hydroxyl group at the non-reducing end. In addition to this CGTases also display hydrolytic activity. Although the hydrolytic activity is very low compared to cyclodextrin synthesis, there are a few examples of CGTases with high residual hydrolytic activity, for instance, CGTases from *Thermoanaerobacterium thermosulfurigenes* EM1(Tabium), *Bacillus* sp. SK 13.002 and the CGTase from *Carboxydocella* sp. (**Paper II**; (Bornscheuer and Kazlauskas 2004, Kelly, Leemhuis et al. 2007). As previously discussed in section 5.1, a crucial structural difference, believed to be the main determinant of cyclization in CGTases, is a loop present in  $\alpha$ -amylases which is responsible for hindering the substrate interactions in -3/-4 subsites. To prove the importance of the loop, five amino acids were inserted in the Tabium CGTase to make a

corresponding extended loop at the donor subsites, that was shown to hamper the cyclization activity severely and converted the enzyme into an *exo*-hydrolase (Leemhuis, Kragh et al. 2003). Moreover, to improve the ratio of cyclization over hydrolysis in GH13 CGTases both site-directed mutagenesis and directed evolution have been applied, with varying success. These mutational studies provided more insights into the molecular aspects that determines the propensity of cyclization as well as the hydrolysis in CGTases.

#### **5.3.1.1 Role of acceptor subsites**

Mutational studies in the acceptor subsites, have proven that the aromatic and hydrophobic interactions in the +2 and +3 subsites are crucial for the balancing of hydrolysis and cyclization reactions. Since these interactions are important for the binding of sugar molecules in the acceptor subsites, their removal facilitated hydrolytic activity while cyclization was significantly decreased (van der Veen, Leemhuis et al. 2001, Leemhuis, Dijkstra et al. 2002, Shim, Kim et al. 2004). Another residue, Ala<sup>230</sup> at +1 subsite also found to be important for the ratio of hydrolysis/cyclization. This was identified via a directed evolution approach and substitution to a valine at this position changed the CGTase into a  $\alpha$ -amylase-like hydrolytic enzyme (Leemhuis, Rozeboom et al. 2003, Kelly, Leemhuis et al. 2007). The mechanism behind this change in the reaction specificities is related to the fact that the presence of valine caused steric hindrance to substrate binding at the +1 subsite. This is supported by the induced-fit mechanism proposed by Uitdehaag et al., that a sugar unit acting as acceptor molecule binds to the respective subsite and can activate the enzyme for the cyclization reaction (Uitdehaag, Van Alebeek et al. 2000).

#### **5.3.1.2 Role of donor subsites**

The above studies explain the critical role of acceptor subsites in determining the propensity of cyclization over hydrolysis, however the donor subsites have been shown to be more involved in modification of cyclodextrin specificities by changing the ratio of  $\alpha$ ,  $\beta$  and  $\gamma$  cyclodextrins. In addition to the donor subsites, the Tyr<sup>195</sup> residue located at a central position is also very important for defining the cyclodextrin specificities (Wind, Uitdehaag et al. 1998, van der Veen, Uitdehaag et al. 2000, van der Veen, Uitdehaag et al. 2000, Leemhuis, Uitdehaag et al. 2002, Nakagawa, Takada et al. 2006, Goh, Mahadi et al. 2009, Li, Zhang et al. 2009).

It is quite evident that improving the cyclization activity in CGTases is not very straight-forward. Nevertheless, by applying directed evolution, a CGTase mutant Ser77Pro was identified by Kelly and co-workers that performed only cyclization with significantly lower amount of hydrolysis (Kelly, Leemhuis et al. 2008). It is noteworthy that the Ser<sup>77</sup> residue that was mutated is located 10 Å from the active site, putting it in the outer region. The structural data of the mutant suggested that

the Tyr<sup>101</sup> residue on the same strand as Ser77Pro underwent conformational changes due to lack of a hydrogen bond that affected the hydrogen bonding to Arg<sup>228</sup>. As a result, Arg<sup>228</sup> attained a new conformation that directly affected the orientation of Glu<sup>258</sup>, the catalytic acid/base. It has been demonstrated by previous studies that binding of an acceptor molecule (except water) could restore the catalytic conformation (Barends, Bultema et al. 2007, Kaper, Leemhuis et al. 2007).

### **5.3.2 Coupling and disproportionation activity**

Two intermolecular transglycosylation reactions, catalysed by the CGTases, are coupling and disproportionation (Fig.6). Both of these transglycosylation reactions have been used in glycosylation of different acceptor molecules with and without sugar moiety (Leemhuis, Kelly et al. 2010). Examples of glycosylated products with improved solubility and biological functions that have been obtained using these reactions are 2-O- $\alpha$ -D-glucopyranosyl-L-ascorbic acid (AA-2G) (Jun, Bae et al. 2001) and alkyl glycosides (using coupling activity) (Svensson, Ulvenlund et al. 2009). In these reactions maltodextrins, cyclodextrins or even starch can be used as donor substrates.

### **5.3.3 Alkyl glycoside as acceptor in coupling reaction**

The coupling activity has been shown to be the desired route for the elongation of alkyl glycosides by using a commercial CGTase (Amano) from Amano Enzyme Inc, (Svensson, Ulvenlund et al. 2009) and later by other CGTases (**Paper I-II**). This reaction involved cyclodextrins as donor and dodecyl- $\beta$ -maltoside (DDM) as acceptor substrates. The physio-chemical properties of these two substrates makes the coupling reaction more complex, as the cyclodextrins are known for making inclusion complexes with hydrophobic moieties and the alkyl glycosides are known to make micelles. As a result, several different reaction species are formed during the elongation process (Zehentgruber, Lundemo et al. 2011).

### **5.3.4 Donor preference for coupling activity**

To utilise the full potential of alkyl glycosides, it is important to extend the carbohydrate head-group. Since  $\alpha$ -cyclodextrin was used as donor in all the previous work on elongation of DDM, CGTases with efficient coupling activity for larger cyclodextrins than  $\alpha$ -cyclodextrin are of great interest. Unfortunately, few CGTases are studied with regard to the alkyl glycoside synthesis using coupling activity; hence little is known about their donor preference.

Previous studies have demonstrated that the cyclization specificity of a CGTase also determined the preference of cyclodextrins as donor in coupling reaction (Jun, Bae et al. 2001). This was, however, not the case for a novel CGTase from the thermophilic organism *Carboxydocella* sp. (**Paper I**). The novel CGTase *CspCGT13* preferentially forms  $\alpha$ -cyclodextrin but has higher coupling and disproportionation activity using  $\gamma$ -cyclodextrin as donor. Two other CGTases used in the comparison study showed the previously demonstrated preference in both cyclization specificity for  $\alpha$ -CD and use of it as preferred donor in the coupling reaction (**Paper I**).

In order to understand what determines the donor preference in the coupling reaction, an amino acid sequence comparison was performed for *CspCGT13*, CGTase from *B. circulans* 251 and sequences of the CGTases that the commercial enzymes originated from (*Thermoanaerobacter* sp. and *Paenibacillus macernas* for Toruzyme 3.0L and Amano enzymes respectively) (Table 2, **Paper I**). Sequence variations were observed at the -3 and -7 subsites. The -7 subsite is largely involved in cyclodextrin specificity for cyclization reaction (Leemhuis, Kelly et al. 2010) but too remote to play any significant role in interactions with the cyclic donor. Therefore, the -3 subsite became the likely candidate for donor specificity in the coupling reaction.

**Table 2:** Sequence comparison of the substrate interacting residues of CGTases based on *Bacillus circulans* 251 (**Paper I**).

Enzyme	Subsite +2	Subsite +1	Subsite -1	Subsite -2	Subsite -3	Subsite -6	Subsite -7
Residue numbering according to CGTase from <i>B. circulans</i> 251	183, 232, 259	194-195, 230, 233	100, 140, 227, 229, 257, 327-328	98	47, 89, 94, 196, 371	167, 179-180, 193	145-147
<i>Bacillus circulans</i> 251 ( $\beta$ )	F, K, F	LY, A, H	Y, H, R, E, HD	H	R, Y, N, D, D	Y, GG, N	SSD
<i>Carboxydocella</i> sp. <i>CspCGT13</i> ( $\alpha/\beta$ )	F, K, F	LF, A, H	Y, H, R, E, HD	H	K, D, S, D, D	Y, GG, N	NQS
<i>Thermoanaerobacter</i> sp. ATCC 53627 ( $\alpha/\beta$ )	F, K, Y	LF, A, H	Y,H,R, E, HD	H	K, D, S, D, D	Y, GG, N	SET
<i>Paenibacillus macernas</i> ( $\alpha$ )	F, K, Y	LY, A, H	Y, H, R, E, HD	H	K, Y, N, D, D	Y, GG, N	SST

Mutational studies in this region showed that the Try<sup>89</sup> residue is important for coupling activity of CGTases. Substitution of this residue to serine in a CGTase from *Bacillus* sp. I-5 had enhanced the coupling activity (Kim, Bae et al. 1997). In another study Try<sup>89</sup>Asp mutant of the CGTase from *B. circulans* 251 also showed increase in the reaction rate for coupling (van der Veen, Uitdehaag et al. 2000). Based on the sequence analysis showed in Table 2, *CspCGT13* has aspartic acid in position 89 and, in accordance with van der Veen et. al. (van der Veen, Uitdehaag et al. 2000), has high coupling activity. However, the origin of

the Amano enzyme *P. macerans* CGTase, has tyrosine in position 89 and showed high coupling activity using  $\alpha$ -CD as donor. This residue was also mutated in *P. macerans*, where the Tyr89Asp mutant showed increased  $\alpha$ -cyclodextrin specificity, but effect of this mutant on coupling activity is not known (Li, Zhang et al. 2009). Hence, at the moment it is not quite clear what determines the donor specificity in coupling reaction.

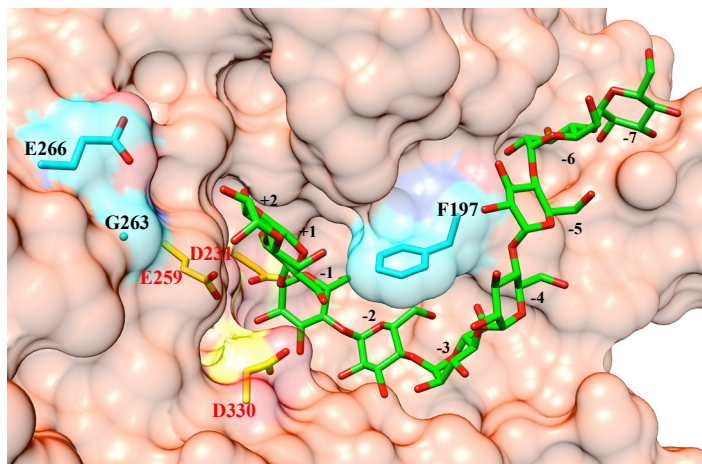
### 5.3.5 Improving the coupling activity

The elongation of alkyl maltosides using  $\gamma$ -CD resulted in a carbohydrate head-group with DP10. Moreover, during this process, products are generated via coupling and disproportionation reactions, see figure 6 for reaction mechanism (**Paper I** and **II**). To produce alkyl glycosides with defined length of the carbohydrate chain, coupling activity is more desired as disproportionation resulted in carbohydrate chain with varying chain length (Svensson, Ulvenlund et al. 2009) (**Paper II**). The *Csp*CGT13 also showed better coupling activity while using methyl- $\alpha$ -glucopyranoside as acceptor (**Paper II**). However, in both cases the disproportionation activity was significant. Thus, to achieve better yield of the elongation process, it is important to improve the reaction specificity for the coupling activity.

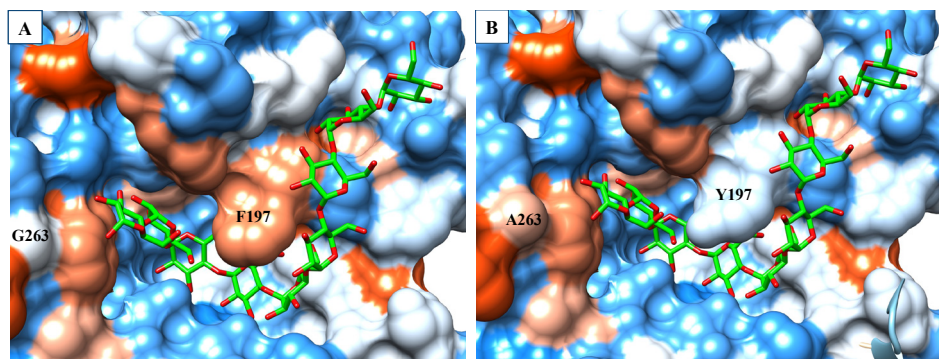
The acceptor substrate in alkyl glycoside elongation is hydrophobic in nature, but the presence of two glucose units in dodecyl- $\beta$ -maltoside improve the solubility of the molecule compared to the dodecyl- $\beta$ -glucoside (DDG) which has very low solubility. Still, to favour the coupling it is important to understand the binding interactions between the alkyl glycoside and the sub-sites, which has not been studied before. The interaction between DDM and enzyme active site have been studied for *Csp*CGT13 in **Paper III** and will be discussed in the following sections.

### 5.3.6 Mutational study of *Csp*CGT13

A striking difference between the Amano CGTase and *Csp*CGT13 was the disproportionation activity which was significantly lower for Amano (**Paper II**) (no disproportionation was observed using 20 mM of DDM, **Paper I**). This enzyme was originally developed for producing cyclodextrins with specificity towards  $\alpha$ -CD which is also supported by **Paper I**. To enhance the cyclodextrin production the hydrophobic interactions in the acceptor subsites are very crucial, as described in section 5.3.1.1.



**Figure 7:** The active site of the modelled *CspCGT13* structure from *Carboxydocella* sp.. The subsites were predicted by overlaying the ligand maltononanose from a co-crystallised structure of *Bacillus circulans* 251 (PDB: 1CXK), shown in green. The catalytic triad shown in yellow and the selected residues for mutations in cyan (Adopted from **Paper III**).

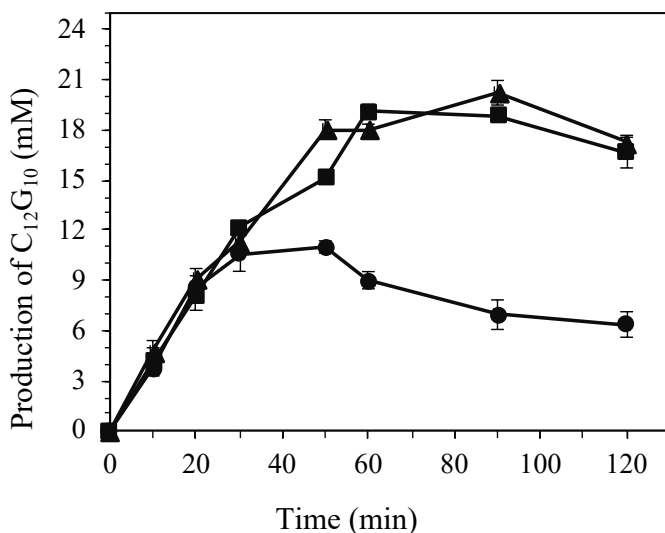


**Figure 8:** Changes in the hydrophobicity surfaces of the active site induced by the mutant F197Y/G263A. (A) represents the active site of the wildtype *CspCGT13* and (B) represents the mutant F197Y/G263A. The hydrophobicity of the surfaces represented according to the Kyte-Doolittle scale (Kyte and Doolittle 1982), from dodger blue for the most hydrophilic to orange red for most hydrophobic (Adopted from **Paper III**).

Based on the sequence analysis and molecular modelling of *CspCGT13*, residues at three different positions Phe<sup>197</sup>, Gly<sup>263</sup> and Glu<sup>266</sup> were chosen for mutagenesis as depicted in Figure 7. The residue 197 located at the centre of the active site cleft, is the most studied residue in CGTases and *CspCGT13* has a phenylalanine (Phe<sup>197</sup>) at this position (Fig. 7). Presence of an aromatic residue at this position is shown to be vital for cyclization reaction and a CGTase with a tyrosine residue at the position 197 showed higher coupling activity (Penninga, Strokopytov et al.

1995). It is hypothesised that substituting Phe to Tyr at the position 197 helps in stabilising the acceptor substrate (DDM) by allowing binding to +1 sub-site (**Paper III**) (Fig. 8B). This extra binding interaction will promote binding of alkyl glycosides with varying-chain lengths including with short hydrophilic head-group, such as DDG. On the other hand, mutation at position 263 is not studied in other CGTases and the glycine residue at this position was found to be conserved among other CGTases (**Paper III**). The sidechain of this residue is oriented in close proximity to the +2 subsite. Changing this residue to alanine is going to increase the hydrophobicity in the acceptor sub-site (Fig. 8A-B). This increased hydrophobicity is important for accommodation of the alkyl-chain of the acceptor molecule (**Paper III**). The glutamic acid at position 266 was also substituted to alanine, to reduce the glucose binding at the potential +3 subsite, shown to be involved in disproportionation activity (van der Veen, Leemhuis et al. 2001).

The results from the mutational study indeed indicate the importance of hydrophobicity at the acceptor subsites in alkyl maltoside elongation reactions by coupling. The double mutant showed that both residues 197 and 263 played a crucial role in the coupling reaction (**Paper III**). The highest coupling activity was achieved for the mutant F197Y/G263A and with molecular modelling it was demonstrated that the G263A interacts with the alkyl chain of dodecyl maltoside and that F197Y interacts with the carbohydrate head-group (**Paper III**) (Fig. 8B).



**Figure 9:** Time course of the production of  $C_{12}G_{10}$  for CspCGT13 and its mutant. The closed circle represents CspCGT13, the closed square for the mutant F197Y/G263A and the closed triangles for the mutant F197Y/G263A/E266A.



Moreover, the time course for the formation of the primary coupling product which in this case is dodecyl-maltodecanoside ( $C_{12}G_{10}$ ), showed significant increase for both the mutant F197Y/G263A and a triple mutant involving these two residues (Fig. 9).

For reduction of the disproportionation activity, the single mutations were not very successful while the double substitutions were more promising (**Paper III**). It is noteworthy to mention that the hydrolysis of  $\gamma$ -cyclodextrins during alkyl maltoside modification makes the interpretation of the product profiles complicated. Hence, the increased disproportionation activity can also be due to increased donor hydrolysis. The mutations had no significant effect on the donor preference and little effect on starch hydrolysis (**Paper III**).

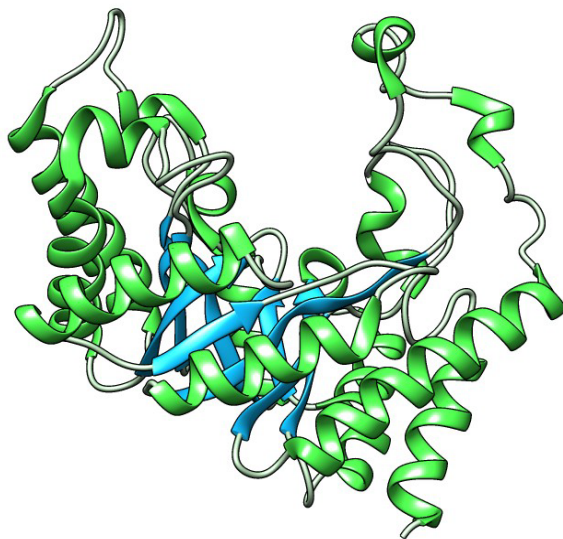
## 6 Transglycosylation by a retaining *endo*-xylanase

Unlike the transglycosylases of GH13 discussed in the previous chapter and Paper I-III, hydrolysis is the predominant function of most retaining GHs from other families, and therefore understanding of the structure-function relationships with regards to improving the ratio of transglycosylation over hydrolysis is quintessential. The broad substrate specificity of the many other retaining GH families makes them more preferred for different transglycosylation applications, explicitly for oligosaccharide synthesis.

To favour the transglycosylation reaction, retaining GHs from various GH families have been investigated in detail using protein engineering. Based on these studies, a few important strategies have been identified which can be adapted to improve the balance between hydrolysis and transglycosylation. The focus of the following chapter will be the strategies involving subsite interactions in the active site of  $\beta$ -retaining GHs, with an emphasis on GH10 *endo*-xylanases and their role in oligosaccharide synthesis.

### 6.1 Endo- $\beta$ -xylanases from GH family 10

Endo- $\beta$ -1,4-xylanases (EC 3.2.1.8) also known as xylanases, cleave the internal glycosidic bonds between two xylopyranosyl units in the xylan backbone to produce xylo-oligosaccharides with varied lengths. They have been classified under different GH families; and xylanases from GH5, GH10 and GH11 are all using the retaining mechanism during catalysis. The catalytic domain of GH5 and GH10 share the TIM barrel fold and belong to clan GH-A whereas GH11 has a  $\beta$ -jelly roll fold and is classified under the GH-C clan ([www.cazy.org](http://www.cazy.org)) (Lombard, Golaconda Ramulu et al. 2013).



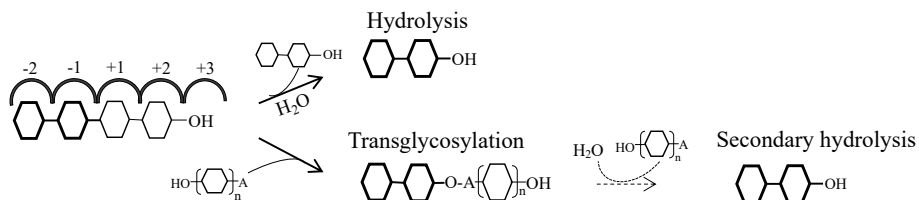
**Figure 10:** Structural representation of a GH10 xylanase from *Cellvibrio mixtus* (PDB:1UQY). The TIM barrel fold is represented with  $\alpha$ -helices in light green colour and  $\beta$ -strands in light blue.

The structure of GH10 *endo*- $\beta$ -xylanases are often modular with a carbohydrate binding module (CBM) attached to the catalytic module (CM) that possesses the  $(\alpha/\beta)_8$  fold (TIM barrel fold) comprising eight  $\alpha$ -helices and  $\beta$ -strands. The arrangements of the  $\beta$ -strands are parallel, flanked by the  $\alpha$ -helices. This representation of the CM is known to resemble the shape of a ‘salad bowl’ with the catalytic site positioned at the narrower end of the barrel (Fig. 10).

### 6.1.1 Active site cleft

The active site of the GH10 *endo*-xylanases is formed by loops at the C-terminal end of the  $\beta$ -strands. The glutamate residues conserved as acid/base and catalytic nucleophile in GH10 is situated on  $\beta$ -strands 4 and 7, respectively (Jenkins, Leggio et al. 1995). The number of subsites involved in the substrate interactions varies between four to six (Pell, Szabo et al. 2004). Like other  $\beta$ -retaining glycoside hydrolases, GH10 *endo*-xylanases also show a well-conserved glycone region (donor subsite), the -1 and -2 subsites. However, in the aglycone region of GH10 *endo*-xylanases (acceptor subsites) the only conserved interacting residues are located in the +1 subsite (White, Tull et al. 1996, Notenboom, Birsan et al. 1998, Ducros, Charnock et al. 2000). Residues conserved in -1, -2 and +1 subsites play important roles in recognition and interactions between the xylopyranosyl moiety and the enzyme during hydrolysis (Charnock, Spurway et al. 1998, Zolotnitsky, Cogan et al. 2004). The less conserved aglycone residues are

however interesting, as they may be important in determining substrate specificities (Solomon, Teplitsky et al. 2007).



**Figure 11:** Schematic illustration of the reactions catalysed by the GH10 *endo*-xylanase RmXyn10A (**Paper IV**).

## 6.2 Catalytic mechanism

Like other retaining enzymes GH10 *endo*-xylanases also catalyse through the double displacement mechanism (Fig. 11). Using this catalytic mechanism, GH10 xylanases can hydrolyse both xylooligosaccharides (XOS) and heteroxylan with and without substitutions (Biely, Vrřanská et al. 1997, Beaugrand, Chambat et al. 2004, Pell, Taylor et al. 2004, Kolenová, Vrřanská et al. 2006). They produce xylooligosaccharides and compared to GH11 xylanases, the produced oligosaccharides are by many authors reported to have a lower degree of polymerization (DP) after hydrolysis of arabinoxylan and glucuronoxylan (Biely, Vrřanská et al. 1997, Vardakou, Katapodis et al. 2003, Pollet, Delcour et al. 2010).

## 6.3 Favouring transglycosylation over hydrolysis

To make the *endo*- $\beta$ -xylanases useful for XOS synthesis of higher DP, the ratio between hydrolysis and transglycosylation needs to be altered. As mentioned in the beginning of this chapter, this can be achieved by using protein engineering. Two major approaches of protein engineering concerning the acceptor and donor subsites are presented below.

### 6.3.1 Improving interactions in acceptor subsites

Binding of the acceptor molecule is influenced by the interactions of aglycone residues and modulation of these residues has been shown to affect the transglycosylation activity. Several studies on  $\beta$ -*endo*-GHs have demonstrated that the aromatic residues play a key role in acceptor affinity and are critical for efficient transglycosylation (Armand, Andrews et al. 2001, Johansson, Brumer et al. 2004, Dilokpimol, Nakai et al. 2011, Rosengren, Häggglund et al. 2012). To elucidate how the acceptor affinity determines the transglycosylation yield in a GH10 xylanase, the aglycone subsites of a xylanase (Xyn10A) from *Pseudomonas cellulosa* were subjected to a mutational study by Armand and co-workers (Armand, Andrews et al. 2001). The findings of that study demonstrated a significant decrease in transglycosylation activity when key residues in the +2 (Asn<sup>182</sup>), +3 (Tyr<sup>255</sup>) and +4 (Tyr<sup>220</sup>) subsites were substituted by alanine. This result indicates that decreased affinity in these regions favour hydrolysis over transglycosylation. It is noteworthy to mention that the effect of aromatic residues may differ depending on the nature of the acceptor molecule (Ochs, Belloy et al. 2013). The studies mentioned above used saccharides as acceptor molecules and the role of tryptophans and other aromatic residues in the aglycone subsites have been considered crucial for oligosaccharide synthesis. For hydrophobic acceptors, like alcohols, the interactions with aglycone subsites may not be similar to those observed for saccharides. It has been shown that substitution of Trp residues did not affect the transglycosylation activity when an alcohol was used as an acceptor (Morrill, Månberger et al. 2018). However, modulating the region can influence the preference for different types of alcohol acceptors.

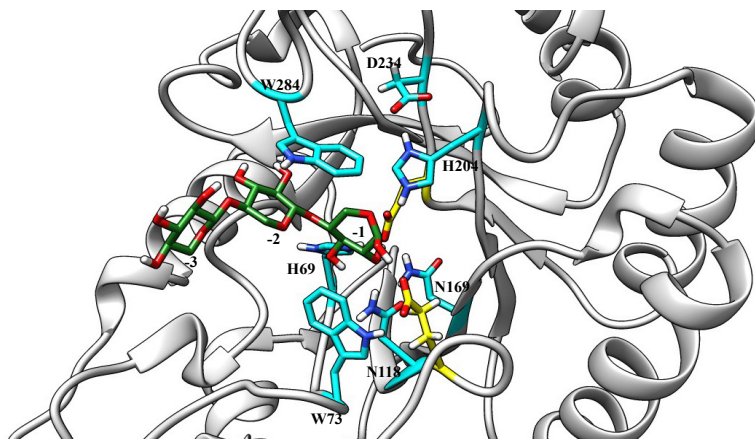
### 6.3.2 Minimizing interactions in donor subsites

The interactions in the glycone (donor) regions of the active site have been studied broadly through protein engineering. However, for GH10 xylanases, this region has been explored for hydrolysis and catalytic function rather than transglycosylation (Moreau, Shareck et al. 1994, Charnock, Lakey et al. 1997, Charnock, Spurway et al. 1998). Recently, site-directed mutagenesis of the conserved residues in -1 subsite in another GH family, an *exo*-acting GH1  $\beta$ -glucosidase, resulted in higher disaccharide yields (Teze, Hendrickx et al. 2013). This could be expanded to GH36, where a similar trend in saccharide synthesis was observed for an  $\alpha$ -galactosidase (Teze, Daligault et al. 2014). Both studies support the hypothesis that a reduction in the interactions in the -1 subsite decreases the stabilisation of the transition states in the retaining mechanism, affecting the hydrolysis. It is reasoned that in GH36 and GH1, transglycosylation involves both -1 and +1 subsites while hydrolysis mainly involves the -1 subsite. Increased transglycosylation have also been reported for other GHs (both *endo* and *exo*-acting) where residues in donor subsite have been subjected to mutation

(Feng, Drone et al. 2005, Aronson, Halloran et al. 2006, Arab-Jaziri, Bissaro et al. 2015).

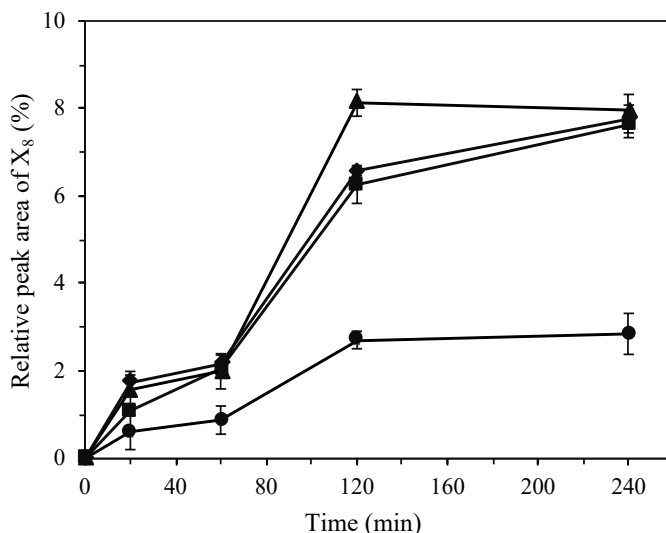
## 6.4 Mutational study of *RmXyn10A\_CM*

The catalytic module of GH10 *endo*-xylanase, *RmXyn10A\_CM* from *Rhodothermus marinus* DSM 4252<sup>T</sup> is known to efficiently hydrolyse xylan and arabinoxylan (Falck, Precha-Atsawan et al. 2013, Salas-Veizaga, Villagomez et al. 2017). Therefore, previous mutagenesis work was focused on manipulating the hydrolytic activity (Aronsson, Güler et al. 2018). Utilisation of *endo*-GHs for oligosaccharide synthesis is, however, quite interesting and the possibility to influence transglycosylation via changes in the -1 subsite was thus explored (Paper IV).



**Figure 12:** Active site of *RmXyn10A\_CM*. The ligand xylootriose is docked into the active site showed in green colour, catalytic residues in yellow and selected residues for mutation are in cyan.

A mutagenesis approach based on sequence conservation was applied to the *RmXyn10A\_CM* aimed to improve the transglycosylation activity. This approach is generic and does not require structural information like the previous studies mentioned above. The mutations were done based on a few criteria: i) substitution of the most conserved residues to their analogues, e.g. Asp into Asn or Tyr into Phe, ii) catalytic residues and residues distant from -1 subsite were excluded, iii) conserved glycines and prolines were not substituted. From the sequence conservation analysis residues at seven different positions were selected for mutagenesis in *Rmxyn10A\_CM*, illustrated in Figure 12.



**Figure 13:** Monitoring of xylooctose ( $X_8$ ) synthesis by *RmXyn10A* and its mutants using HPAEC-PAD. The closed circles refer to the wildtype *RmXyn10A*, closed squares to H69N, closed triangles to N118T and closed diamonds to W284H.

#### 6.4.1 Improved transglycosylation activity

The transglycosylation ability of *RmXyn10A*\_CM and its mutants were evaluated by following the XOS formation. In this reaction xylotetraose ( $X_4$ ) was used as donor and acceptor. The findings from this investigation unveiled that mutations of three conserved residues (His69Asn, Asn118Thr and Trp284His) resulted in significantly improved production of XOS compared to the wild type (**Paper IV**) (Fig. 13). The work on structural analysis of *RmXyn10A*\_CM by Aronsson and co-workers (Aronsson, Güler et al. 2018), reported that His<sup>69</sup>, Asn<sup>118</sup> (His<sup>617</sup> and Asn<sup>666</sup>, following the numbering of the full length enzyme was used in the study by Aronsson et al.) make interactions in the -1 subsite through hydrophobic interaction and hydrogen bonding, respectively. The Asn<sup>118</sup> residue has previously been studied for the xylanase from *Pseudomonas cellulosa* (XYLA), where substitution of the Asn residue by alanine resulted in significant decrease in hydrolysis. However, no transglycosylation data was available for the mutant (Charnock, Lakey et al. 1997).

Hydrogen bonding interactions of His<sup>69</sup> and Asn<sup>118</sup> with -1 subsite is highly conserved among GH10 members and have influence on the protonation state of catalytic acid/base residue (Pell, Taylor et al. 2004). Similar interaction was also seen in *RmXyn10A*\_CM and *p*-Nitrophenyl-xylotrioxide (*p*NP- $X_3$ ) hydrolysis data of the mutants (His69Asn and Asn118Thr) showed 23-fold decrease in activity compare to the wild-type (**Paper IV**). The high transglycosylation

activity for these two mutants indicates that they might have potentially altered the capacity of Glu<sup>119</sup> to abstract proton from a water molecule, hence affecting the stabilisation of the transition state (Teze, Daligault et al. 2014).

The conserved residue mutant Trp284His, which is located at the top of the donor subsites, showed a similar loss of hydrolytic activity as mentioned above. The corresponding residue, numbered as Trp<sup>274</sup> (equivalent to the residue Trp<sup>284</sup> in *RmXyn10A\_CM*) in the xylanase (Xyl10A) from *Streptomyces lividans* was shown to make stacking interactions with Arg<sup>275</sup> and was involved in interactions with the -1 subsite (Ducros, Charnock et al. 2000). In *RmXyn10A\_CM*, this residue is turned 180°, making it exposed to the solvent and this might have impact on the interactions with -1 subsite (Aronsson, Güler et al. 2018). Indeed, this mutant showed no significant loss of hydrolytic activity against *p*NP-X<sub>2</sub> (unpublished data), clearly indicating strong interaction with +1 subsite, explaining the transglycosylation activity of this mutant.





## 7 Hydrolysis by $\beta$ -glycosidases from GH family 3

Transglycosylation by retaining GHs and TGs has been the focus of the previous chapters. However, the following chapter will focus on how the structurally related enzymes (a single GH family) gain different activities and domain compositions. In this case, hydrolysis reactions by *exo*- $\beta$ -glycosidases from family 3, have been studied. In chapter 2 the reaction mechanism and structural features that govern the properties of retaining GHs has been discussed. The discussion in the following sections will be dedicated towards their structural features, substrate specificity and how they are distributed in the family.

### 7.1 Glycoside hydrolase family 3 (GH3)

GH3 is one of the largest families in the CAZy database (Lombard, Golaconda Ramulu et al. 2013) containing over 28000 entries, among which the number of biochemically characterised GH3 enzymes are 313 (December 27, 2019; [www.cazy.org](http://www.cazy.org)). GH3 sequences are distributed in different *domains* (bacteria, eukaryota, and archaea), in particular most of the sequences originated from different groups of bacteria, plants and fungi. The family comprises enzymes demonstrating the following activities:  $\beta$ -glucosidase,  $\beta$ -xylosidase,  $\alpha$ -L-arabinofuranosidase,  $\beta$ -*N*-acetyl-glucosaminidase,  $\beta$ -*N*-acetyl-glucosaminide phosphorylases, *exo*-1,3- $\beta$ -glucosidase, *exo*-xyloglucanase and other (Davies and Henrissat 1995, Harvey, Hrmova et al. 2000).

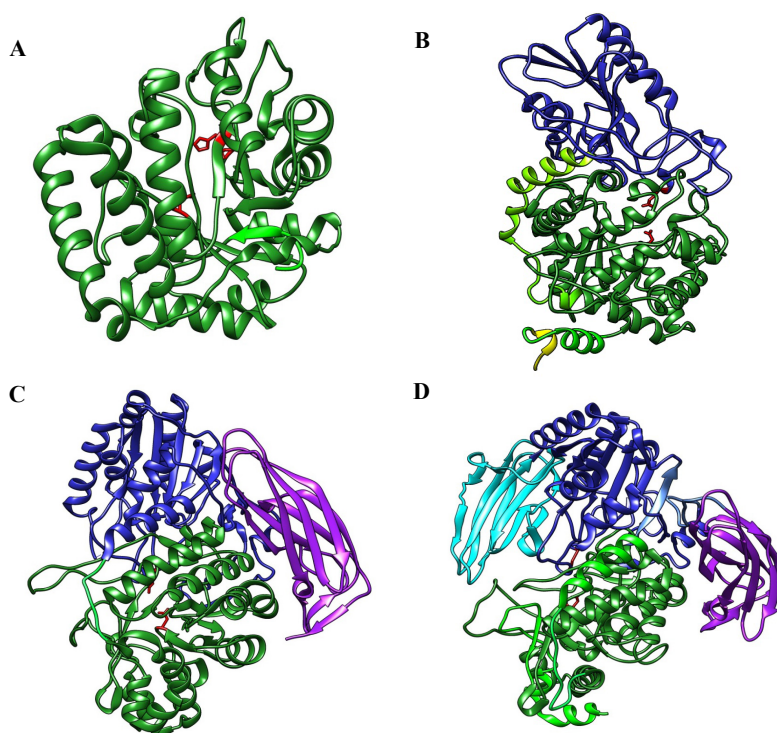
Members of this family are retaining GHs and capable of hydrolysing the terminal glycosidic bond in the non-reducing end of a number of  $\beta$ -linked glycoconjugates via the double-displacement mechanism as illustrated in Figure 1.

Enzymes from GH3 have been studied for their roles in various biological functions including biomass degradation, cell-wall remodelling and defence against pathogens (Faure 2002, Johnson, Fisher et al. 2013). This family gained more attention in recent years for its application in degradation of renewable resources for biofuel production (Li, McCorkle et al. 2009). Thus, GH3 members

from *Rhodothermus marinus* DSM 4253 are of great interest for biomass degradation as their temperature optima ranges from 60-80°C (**Paper V**).

## 7.2 Overall structure and domain organisation of GH3

In GH family 3 the number of domains and their arrangement vary significantly. The first protein structure of GH family 3 was reported in 1998 which was a  $\beta$ -glucan *exo*-hydrolase from barley called *HvExoI*, comprised of two domains (Varghese, Hrmova et al. 1999). A decade later two more structures of GH3 were published, the first three-domain  $\beta$ -glucosidase *TnBgl3B* from *Thermotoga neapolitana* (Pozzo, Pasten et al. 2010) and the four-domain  $\beta$ -glucosidase *KmBglII* from *Kluyveromyces marxianus* (Yoshida, Hidaka et al. 2010). More structures of GH3 enzymes have been reported in recent years ([www.cazy.org](http://www.cazy.org)).



**Figure 14:** Structures of *exo*-glycosidases from GH family 3, represented in ribbon diagrams. Active site residues are shown as red sticks. (A) VcNagZ (PDB:1Y65), (B) *HvExoI* (PDB:1EX1), (C) *TnBgl3B* (PDB:2X41) and (D) DesR (PDB:4I3G). Domain 1 is coloured in green, domain 2 coloured in blue, FnIII domains are coloured in purple and the PA14 domain coloured in cyan.

The overall structure of the two most common domains in GH3 are the  $(\alpha/\beta)_8$  barrel of domain 1 and  $(\alpha/\beta)_6$  sandwich of domain 2 (Fig. 14A-D). The active site is located at the top of the barrel, formed as a pocket shaped between domain 1 and 2, except for the  $\beta$ -*N*-acetylglucosaminidase, which will be discussed separately below. The catalytic nucleophile residue (Asp) is situated in the first domain and the acid/base (Glu) in the second domain. The additional C-terminal FnIII domain is composed of a  $\beta$ -sandwich structure (Fig. 14C-D). The functionality of this domain is not well understood, and there is no interaction between the active site cleft and FnIII domain. Another novel third domain has also been reported for the *exo*-glucanase ExoP from *Pseudoalteromonas* sp. BB1 interacting with domain 1 and involved in the enzyme activity (Nakatani, Cutfield et al. 2012). However, the fourth  $\beta$ -barrel domain PA14 present in GH3 members is clearly involved in the substrate accommodation through loop interactions (Fig. 14D). The second crystal structure of a  $\beta$ -glucosidase DesR from *Streptomyces venezuelae* containing the PA14 domain was published in 2013. In DesR the domain is rotated by 116° compared to the KmBglI (Zmudka, Thoden et al. 2013). Enzymes with this domain showed preference for smaller glycoconjugates and may also be involved in other biological unknown function (Yoshida, Hidaka et al. 2010, Zmudka, Thoden et al. 2013). The  $\beta$ -*N*-acetylglucosaminidases (NagZs) form a unique group within GH3. They are consisting of domain 1 and 2, but a few exceptions are also present such as GH3 NagZs from Gram-negative bacteria GH3 that only consist of domain 1 (Fig. 14A).

## 7.3 Active site architectures

The topology of the active site in *exo*-glycosidases in GH3 is of the pocket shaped type (Fig. 2A) and known to hydrolyse different lengths of substrates. Thus, active site architecture is vital in understanding the substrate specificity of individual enzymes in GH3. As mentioned earlier, the active site in GH3 is made up of both domain 1 & 2 while for GH3  $\beta$ -*N*-acetylglucosaminidases it is only made up of domain 1. The catalytic residues in *HvExoI* were Asp<sup>285</sup> (nucleophile) and Glu<sup>491</sup> (acid/base) and have been found to be conserved for other members of GH3, except the NagZs. The GH3 enzyme NagZs utilises an Asp<sup>227</sup>-His<sup>229</sup> dyad on the loop e of domain 1 as the acid/base (Vocadlo, Mayer et al. 2000, Litzinger, Fischer et al. 2010).

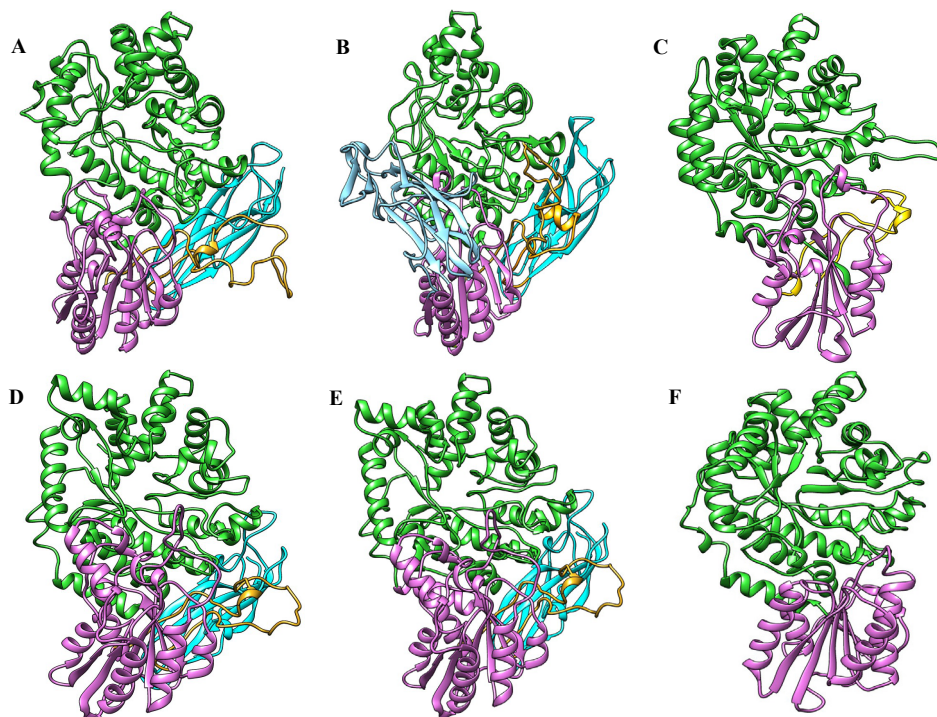
GH3 members have a well-defined glycone (-1 subsite) and an aglycone (+1) subsite in the active site pocket (Fig. 3) that forms interactions with substrates leading to hydrolysis. However, the residues interacting at the -1 subsite are more conserved compared to those in the +1 subsite in GH3 enzymes, which has been found to be influenced by flexible loops (Varghese, Hrmova et al. 1999, Pozzo, Pasten et al. 2010, Yoshida, Hidaka et al. 2010). The active site residues

interacting with the -1 subsite in ExoP are identical to *HvExoI* (Nakatani, Cutfield et al. 2012). The difference between these two enzymes and *TnBgl3B* is a His<sup>164</sup> that interacts with -1 subsite through hydrogen bonding and a Trp<sup>243</sup> and Met<sup>207</sup> through van der Waals interaction (Pozzo, Pasten et al. 2010). Similar architecture was found in *KmBglI* and DesR both containing the PA14 domain (Yoshida, Hidaka et al. 2010, Nakatani, Cutfield et al. 2012).

In the +1 subsite the interactions vary between enzymes, for example, at the +1 subsite *HvExoI* displayed a pair of tryptophan residues (Trp<sup>286</sup> and Trp<sup>434</sup>) facing each other in a hydrophobic clamp. This conformation allows flexibility that affects the accommodation of substrates in the active site; hence contributing to the broad substrate specificity (Varghese, Hrmova et al. 1999, Hrmova and Fincher 2001, Hrmova, De Gori et al. 2004, Hrmova and Fincher 2007). Similar residues were found in ExoP, although the arrangement was antiparallel. Moreover, an additional Trp<sup>494</sup> was suggested to be a part of +2 subsite (Nakatani, Cutfield et al. 2012). The architecture of the +1 subsite is remarkably different in *KmBglI* due to the loops extending from the PA14 domain that cover the catalytic pocket to form the +1 subsite. The Phe<sup>508</sup> residue in the PA14 loop is important for the catalytic activity of the *KmBglI* (Yoshida, Hidaka et al. 2010). However, no substrate interaction for the PA14 domain (which was rotated by 116°) was found in DesR (Zmudka, Thoden et al. 2013).

**Table 3: Sequence comparison of potential substrate interacting residues in  $\beta$ -glycosidases from *Rhodothermus marinus* DSM 4253 with closely related characterised GH3 enzymes. The gaps are indicated as ‘-’**

Enzyme	Subsite -1				Subsite +1					
<i>RmBgl3A</i>	D110	G218	A428		Y287	Y614	G431	-	R606	F254
<i>Elizabethkingia meningoseptica</i> AAB66561.1	D71	G179	S387		Y248	E587	S390	-	R572	F215
<i>RmBgl3B</i>	D71	R178	S380		W246	Y703	V383	-	S682	Y213
<i>Streptomyces venezuelae</i> – DesR, PDB: 4I3G	D98	R206	S410		W274	Y691	V413	-	D670	Y241
<i>RmBgl3C</i>	D121	G234	T450		Y311	-	W453	-	-	Y278
<i>Pseudoalteromonas</i> sp. BB1 – ExoP, PDB: 3F93	D136	G252	S460		W321	-	W463	-	-	F288
<i>Hordeum vulgare</i> – ExoI PDB: 1EX1	D95	G217	T431		W286	-	W434	-	-	Y253
<i>RmXyl3A</i>	E123	G237	S446		W307	-	P449	Y545	E648	Y274
<i>RmXyl3B</i>	E125	G239	S448		W309	-	P451	W547	E650	Y276
<i>Caldanaerobius polysaccharolyticus</i> – Xyl3A, AFM44649.1	E108	G218	A429		Y288	W631	C432	C524	G627	Y255
<i>RmNag3</i>	D118	R186	D227	H229	R60	R126	R306			
<i>Streptomyces thermoviolaceus</i> – NagA, BAA32403.1	D164	R232	D274	H276	R80	R172	G352			



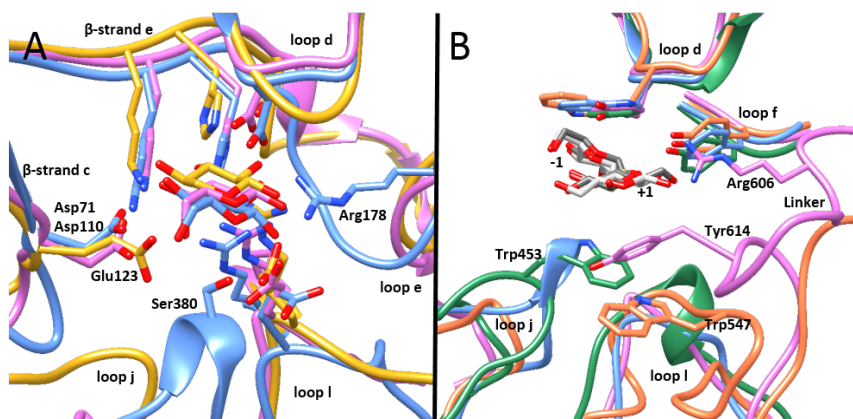
**Figure 15:** Homology models of the six GH3  $\beta$ -glycosidases from *Rhodothermus marinus* DSM 4253. The modelled structures are represented by ribbon diagrams: (A) *RmBgl3A*, (B) *RmBgl3B*, (C) *RmBgl3C*, (D) *RmXyl3A*, (E) *RmXyl3B* and (F) *RmNag3*. Domain 1 shown in light green colour, domain 2 in light purple, FnIII in cyan, PA14 in light sky blue and the linker between domain 2 and FnIII in gold. The  $\beta$ -lactamase domain of *RmNag3* is not represented here (**Paper V**).

## 7.4 Exo- $\beta$ -glycosidases from *Rhodothermus marinus*

During characterisation of the six GH3  $\beta$ -glycosidases from *R. marinus* DSM 4253, their domain organisation and structural features were investigated by homology modelling (**Paper V**). These enzymes include three  $\beta$ -glucosidases (*RmBgl3A*, B and C), two  $\beta$ -xylosidases (*RmXyl3A* and B) and a  $\beta$ -*N*-acetylglucosaminidase (*RmNag3*). The modelled structures of these enzymes displayed the most common domain arrangements as mentioned in section 7.2. The enzyme *RmBgl3C* represents the classical two-domain structure (Fig. 15C), while an FnIII domain was found in *RmBgl3A*, *RmBgl3B*, *RmXyl3A* and *RmXyl3B* (Fig. 15A-B, D-E). The PA14 domain of *RmBgl3B* is situated on top of the barrel in contact with domain 2 (Fig. 15B) and has the similar arrangement as DesR (Zmudka, Thoden et al. 2013) (**Paper V**). The *RmNag3* from *R. marinus* DSM 4253 has a  $\beta$ -lactamase domain in addition to domain 1 and 2 which was not modelled (Fig. 15F) (**Paper V**).

### 7.4.1 Active site

The active sites of the modelled  $\beta$ -glycosidases from *R. marinus* are pocket shaped and involve both domain 1 and 2 like the other members of the family, although with the only exception being *RmNag3*, as discussed in section 7.3. However, the shape of the pocket varies among the enzymes (**Paper V**, supplementary information). For *RmBgl3B* the active site pocket is very deep and narrow, and the PA14 domain as well as a long loop **g** covers the surface over the active site. For *RmBgl3A*, the linker between domain 2 and the FnIII domain on the opposite chain is involved in shaping the active site. This linker makes the active site deeper on one side, compared to the active site of *RmBgl3C*, *RmXyl3A* and *RmXyl3B*. On the other hand, the active site arrangement of *RmNag3* was built up by domain 1 (**Paper V**) and will be discussed in section 7.4.1.1.



**Figure 16:** Comparison of active sites of the  $\beta$ -glucosidase and  $\beta$ -xylosidases. Substrate interacting residues are shown for (A) subsite -1 and (B) subsite +1. Superimposition of modelled structures of *RmBgl3A* (pink), *RmBgl3B* (blue), *RmBgl3C*, *RmXyl3A* (yellow) and *RmXyl3B* (orange). Ligand represented as cellobiose (dark grey) and laminarbiose (light grey) in subsite -1 and +1 from PDB 1IEY and 1J8V respectively (adopted from **Paper V**).

Although variations were observed in the active site arrangement, both  $\beta$ -glucosidases and  $\beta$ -xylosidases showed well-defined +1 and -1 subsites (Fig. 16A-B) (**Paper V**). An overall comparison of subsite interacting residues for the enzymes from *R. marinus* DSM 4253 is provided in table 3. The conserved -1 subsite was typical of GH3 with a few significant differences, as displayed in Figure 16A. The catalytic nucleophile on  $\beta$ -strand **g** and the catalytic acid/base on loop **I** in domain 2 showed strict conservation in positions corresponding to those in other GH3-structures (**Paper V**). A key difference observed between the modelled structures in **Paper V** involves the  $\beta$ -xylosidases and  $\beta$ -glucosidases: a Glu residue was observed in *RmXyl3A* and B, while an Asp was found in the

corresponding position in the  $\beta$ -glucosidases (*RmBgl3A,B* and *C*), interacting with the respective sugar in the -1 subsite (Fig. 16A). Interestingly, this difference was conserved in the closest relatives in the respective enzyme group (Varghese, Hrmova et al. 1999, Han, Agarwal et al. 2012, Nakatani, Cutfield et al. 2012, Zmudka, Thoden et al. 2013) (**Paper V**).

The +1 subsite investigation revealed that both  $\beta$ -xylosidases and  $\beta$ -glucosidases from *R. marinus* DSM 4253 have an aromatic residue on top of the +1 subsite on loop **d** (Fig. 16B). Moreover, another aromatic residue was also located under the +1 subsite but only for the xylosidases, *RmBgl3A* and *RmBgl3C*, potentially sandwiching the sugar unit. However, the location of this residue is not conserved in the respective enzymes (Fig. 16B). It is noteworthy to mention that the second aromatic residue is a Trp in *RmBgl3C* (Trp<sup>453</sup>) and shown to be conserved in *HvExoI* and *ExoP* as part of the hydrophobic clamp, Table 3 (**Paper V**).

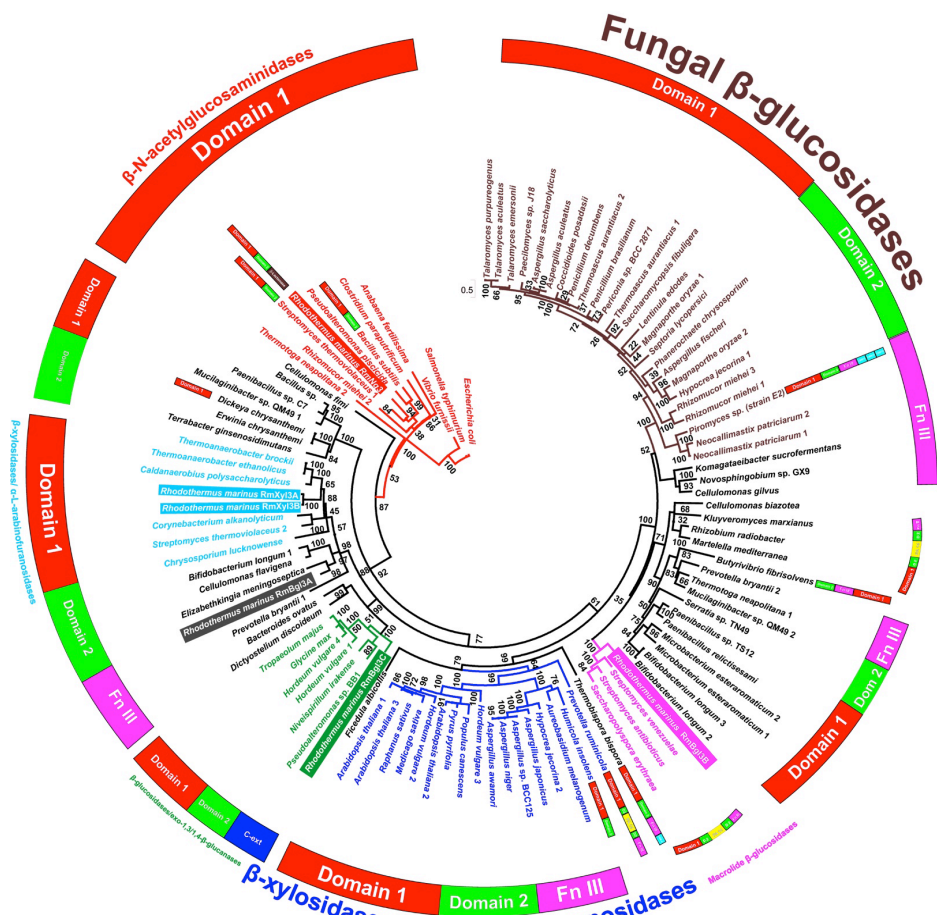
#### 7.4.1.1 $\beta$ -N-acetyl-glucosaminidases

GH3  $\beta$ -N-acetyl-glucosaminidases also have well-defined -1 and + 1 subsites with conserved catalytic residues. Similar to other members, Lys<sup>216</sup>, His<sup>217</sup> and Asp<sup>118</sup> were found interacting with GlcNAc in subsite -1 (Vocadlo, Mayer et al. 2000). More insight into +1 interactions was obtained with a complex of GlcNAc and L-Ala-1,6-anhydroMurNAc in NagZ from *Pseudomonas aeruginosa* where the side-chain of Arg<sup>70</sup> interacted through hydrogen bonding to the peptide unit in subsite +1 (Acebrón, Mahasenan et al. 2017). In *RmNag3* all residues involved in the substrate binding were conserved in the closely related GH3 NagA from *Streptomyces thermoviolaceus* (Table 3). (**Paper V**).

#### 7.4.2 Evolutionary relation with GH3 family

According to Cournoyer and Faure, the GH3 family was divided into three sub-families AB, AB' and AB'' that are further classified into clusters and sub-clusters (Cournoyer and Faure 2003). The AB refers to the domain 1 and 2, B' to the truncated domain 2. The B'' refers to the domain 1 in  $\beta$ -N-acetyl-glucoseaminidases. To understand the evolutionary relationship with respect to the GH3 enzymes from *R. marinus* DSM 4253, a new phylogenetic analysis was described in **Paper V**.





**Figure 17:** Phylogenetic relationship of GH family 3 enzymes from *Rhodothermus marinus* DSM 4253. The maximum likelihood phylogenetic tree was calculated using amino acid sequences of biochemically characterized GH3 enzymes together with six  $\beta$ -glycosidases from *R. marinus* DSM 4253 (highlighted in coloured boxes). Sequences belonging to a particular group have identical colour and the schematic representation of domain organization is shown in a circle above the tree (adopted from **Paper V**).

Based on the maximum likelihood phylogenetic tree, the GH3 family was divided into three evolutionary lineages (Fig. 17). The first lineage includes  $\beta$ -*N*-acetylglucosaminidases, which is the deepest-rooted group in the family. As can be seen in Figure 17, it is separated from other GH3 members and further divided into two sub-groups based on domain numbers. *RmNag3* clusters with the lineage of  $\beta$ -*N*-acetylglucosaminidases. In addition to domain 1 and 2 the *RmNag3* also contains an additional  $\beta$ -lactamase domain (**Paper V**).

The second lineage is divided into three sub-groups comprised of enzymes from plants and bacteria. The xylosidases (*RmXyl3A* and *B*) are clustered with the

sub-group of thermostable multifunctional  $\beta$ -xylosidases, whereas *RmBgl3A* is part of a small cluster made up of bacterial  $\beta$ -glucosidases (Fig. 17). These two sub-groups have similar domain arrangement including domain 1, and FIII. However, the third sub-group consists of enzymes with two domain architecture with C-terminal extension and *RmBgl3C* cluster with these enzymes (**Paper V**). The last lineage is formed by two sub-groups originated from fungi, plants and bacteria. The sub-group consists of bacterial macrolide/ $\beta$ -glucosidases with additional domain PA14 domain (**Paper V**), *RmBgl3B* cluster with the bacterial macrolide  $\beta$ -glucosidases (Fig. 17).

### 7.4.3 Substrate specificities

The GH3 *exo*-acting  $\beta$ -glucosidases have been shown to be capable of removing a single sugar unit from the non-reducing end of a broad range of  $\beta$ -glucans and its oligosaccharides, as well as aryl  $\beta$ -glycosides (Hrmova, Harvey et al. 1996, Hrmova and Fincher 1997, Zverlov, Volkov et al. 1997) (**Paper V**). The multifunctional xylosidases of GH3 are known to hydrolyse,  $\beta$ -xylosides,  $\alpha$ -L-arabinofuranosides and  $\beta$ -glucosides (Mai, Wiegel et al. 2000, Lee, Hrmova et al. 2003, Gao and Wakarchuk 2014) (**Paper V**). When it comes to the linkage specificity and chain length, GH3  $\beta$ -glucosidases are more diverse than  $\beta$ -xylosidases (**Paper V**).

The members of family 3 with the broadest reported substrate specificity are *HvExoI* & II, that are able to hydrolyse  $\beta$ -1,2-,  $\beta$ -1,3-,  $\beta$ -1,4-, and  $\beta$ -1,6-linkages (Hrmova and Fincher 1997). The  $\beta$ -glucosidases *RmBgl3A* and B showed specificity for  $\beta$ -1,4-linkage while the *RmBglC* has more preference towards laminarioligosaccharides (**Paper V**). *Exo*-glucosidases from marine bacteria often show higher specificity for  $\beta$ -1,3-linkage due to its presence in laminarin, a polysaccharide present in brown algae (Zverlov, Volkov et al. 1997, Nakatani, Cutfield et al. 2012). The xylosidases, *RmXyl3A* and *RmXyl3B* were bi-functional and active on xylo- and cellooligosaccharides (**Paper V**). Another bi-functional  $\beta$ -xylosidase (*CpXyl3A*) with similar substrate specificity from GH3 has been characterised from *Caldanaerobius polysaccharolyticus* which is part of a gene cluster for xylan utilisation in the bacterium (Han, Agarwal et al. 2012). However, no endo-activity has been reported for *CpXyl3A* which is the case for *RmXyl3B*, that also showed endo-xylanase activity, indicating diverse functionality in the host bacterium, further discussed in section 7.4 and **Paper V**.

Unlike other members of GH3,  $\beta$ -*N*-acetyl-glucosaminidases are highly selective for *N*-acetyl- $\beta$ -D-glucosamine (GlcNAc). They are active on peptides like, 1,6-anhydroMurNAc for Gram-positive and GlcNAc-MurNAc for Gram-negative bacteria. In both cases they remove a GlcNAc from the peptides (Cheng, Li et al. 2000, Litzinger, Duckworth et al. 2010). A few bi-functional  $\beta$ -*N*-acetyl-

glucosaminidases have been characterised from the family. Nag3 from *Cellulomonas fimi* is known as a  $\beta$ -*N*-acetylglucosaminidase/ $\beta$ -glucosidase and now assigned as a phosphorylase (Mayer, Vocadlo et al. 2006, Macdonald, Blaukopf et al. 2015). Another example is Hs1941 from *Herbaspirillum seropedicae* SmR1 (Ducatti, Carroll et al. 2016), without the phosphorylase activity. It is interesting to compare the substrate specificity of RmNag3 with other characterised enzymes demonstrating *N*-acetyl-glucosaminidase activity, as it was able to hydrolyse chitooligosaccharides by removing a GlcNAc from the non-reducing end and as well did not show any bi-functional activity (**Paper V**). The activity towards chitooligosaccharides indicates its potential function in degradation of chitin. However, presence of the  $\beta$ -lactamase domain suggests stronger association with peptidoglycan turnover as the degradation products have been shown to function as inducers for  $\beta$ -lactamase (Hobel, Hreggvidsson et al. 2005, Zeng and Lin 2013).

## 7.5 A putative polysaccharide utilising locus (PUL) for xylan utilisation in *Rhodothermus marinus*

*R. marinus* is a marine Gram-negative thermophilic bacterium, previously classified under the phylum *Bacteroidetes*, but recently reclassified under *Rhodothermaeota* phyl.nov. (Munoz, Rosselló-Móra et al. 2016). The type species *Rhodothermus marinus* DSM 4252<sup>T</sup> was isolated from a coastal shallow hot spring in Isafjardardjup, on the northwest coast of Iceland. *R. marinus* is aerobic heterotrophic and known to produce a broad spectrum of thermostable enzymes (Spilliaert, Hreggvidsson et al. 1994, Karlsson, Bartonek-Roxå et al. 1997, Crennell, Hreggvidsson et al. 2002, Karlsson, Abou-Hachem et al. 2002, Hobel, Hreggvidsson et al. 2005). In the CAZy database, four different strains of *R. marinus* are represented, all isolated from marine hot springs. For these strains the whole genome sequence is available, and annotations are done for putative glycoside hydrolases. According to the database, each of these strains of *R. marinus* harbour a wide range of GHs, mostly dominated by GH family 3, 13 and 130.

The strain *R. marinus* DSM 4253 that has been discussed in **Paper V** was isolated from the same location as the type strain DSM 4252<sup>T</sup>. Both strains DSM 4252<sup>T</sup> and DSM 4253 contain six genes encoding glycoside hydrolases from family 3, and their corresponding gene clusters are identical. Both of the GH3  $\beta$ -xylosidases characterised from DSM 4253 are found to be involved in a putative PUL for xylan utilisation, which consists of six GHs: two *endo*-xylanases from GH10, two from GH3 (*RmXyl3A* and *B*) and further two uncharacterised GHs

from family 43 and 67 (**Paper V**). In order to understand the mechanism in this putative PUL more studies are required.

# Concluding remarks

In this thesis, glycoside hydrolases from thermophilic bacteria have been isolated and cloned using a metagenomic library and the genomic DNA. They have been demonstrated to be very useful biocatalysts in different biorefinery processes, capable of synthesising surfactants (alkyl glycosides) and oligosaccharides (XOS). The first part of the thesis discusses transglycosylation by retaining glycoside hydrolases and their applications in synthesis, whereas the last part addresses hydrolysis. To expand the understanding of the transglycosylation mechanism and the potential factors determining the transferase and hydrolase activity in glycoside hydrolases, GHs classified both as transglycosylases and as *endo*-acting hydrolases were studied here for two different applications.

For cyclodextrin glucanotransferase (CGTase), the focus is on the elongation of the glycosidic part of alkyl glycosides by using coupling activity. A novel CGTase, *CspCGT13* from *Carboxydocella* sp. is characterized. The data obtained (Paper I) showed significant coupling activity with  $\gamma$ -cyclodextrin as donor, which is useful for the diversity in producing alkyl glycosides of defined length. *CspCGT13*, however, showed better coupling activity with other acceptor molecules (Paper II), indicating poor interaction of the longer alkyl glycoside acceptor molecule. Hence, *CspCGT13* was engineered to improving the acceptor interactions at the active site (Paper III). A variant of *CspCG13* with substitution of two amino acids near the +2 acceptor subsite showed significant improvement in coupling activity. Furthermore, with the help of molecular modelling we could demonstrate how a hydrophobic residue near acceptor subsites could potentially improve stabilisation of the alkyl chain of the acceptor substrate (Paper III). Although, the coupling activity was improved, the novel CGTase still displayed relatively high hydrolytic activity compared to other CGTases. However, no mutational study was undertaken to eliminate the hydrolytic activity. The sequence comparison with other commercial CGTases also indicates that the -3 subsite may be a key position for determining donor specificity. Further investigations are required to verify this.

The study of the *endo*-acting glycoside hydrolase did not involve any surfactant modification, but instead deals with oligosaccharide synthesis. Numerous studies have focused on limiting the hydrolytic activity of glycoside hydrolases and thus improving the transglycosylation yields using a wide range of acceptor substrates. Most of these studies used protein engineering to reduce hydrolysis in GHs, either

through directed evolution or rational design. In this thesis a sequence-based generic approach has been successfully applied to enhance the transglycosylation activity of an *endo*-xylanase catalytic module, (*RmXyn10A\_CM*) from GH family 10 (Paper IV). The mutational study of *RmXyn10A\_CM* from *Rhodothermus marinus* DSM 4252<sup>T</sup> resulted in identification of three conserved residues in the GH10 family that were crucial for the modulating the activity towards a higher transglycosylation ratio for xylooligosaccharide synthesis. To fully explore the potential of the mutants more investigations are needed, especially trials with different types of acceptor molecule would be highly interesting. In addition, the thesis covers the *exo*- $\beta$ -glycosidases from GH3. Six thermostable GH3 enzymes from *Rhodothermus marinus* DSM 4253 have been characterised (Paper V), aiming towards understanding the structure function relationship of these enzymes and their distribution in the host organism. All the  $\beta$ -glucosidases and xylosidases showed broad substrate specificities compared to the  $\beta$ -*N*-acetyl-glucosaminidase (*RmNag3*). Sequence analysis and additional homology modelling displayed variations in the domain organisation among the enzymes. A new  $\beta$ -lactamase domain was also identified for the *RmNag3* which has not been reported previously. Evolutionary relationships of all six enzymes showed that they clustered into three major lineages of GH3 which gives more insights on impact of evolution of enzyme activity. Also, the gene cluster analysis of *Rhodothermus marinus* DSM 4253 and its comparison to the reference strain DSM 4252<sup>T</sup> unveiled a putative polysaccharide utilization locus (PUL) for xylan utilization that include the GH3 xylosidases. This study provides more information about the different roles of different GH3 enzymes isolated from a single microorganism. This study contributes to fundamental understating on how domain structure and active site conservation dictates the activity profiles of structurally related enzymes.

# Acknowledgements

I received great supports and help from many people during my doctoral thesis, and I am very grateful for that. Although I am not very good at expressing my feelings, so, please add the power of infinity on my expressed gratitude.

The person without whom this thesis would not be possible is my supervisor Prof. Eva Nordberg Karlsson. Thank you, Eva, for believing in me and allowing me to work on different projects during my PhD studies. I consider myself very lucky to have you as my Supervisor. You have been more of a mentor during my doctoral studies. You have always encouraged me to explore new ideas and work independently. I sincerely appreciate all your support throughout my time here at the Division.

To my co-supervisor Prof. Patrick Adlercreutz, thank you very much for your invaluable contributions to my work as a PhD student. You have always been there when I need advice and suggestions.

To Paula thank you for turning my thesis into a nice book.

I had the privilege to work with many great scientific minds as my co-authors through different collaborations. To Guðmundur and Ólafur, thank you for all the fruitful collaborations we had with Matis. I always enjoyed our discussions during meetings and conferences. You both have been very kind and patient with me.

Pontus and Younis, thank you for introducing me to the world of surfactant and having smooth collaboration with me.

Sincere gratitude to my co-authors, Štefan Janeček, Anna Månberger, Javier A. Linares-Pastén, Marek Gabriško, Andrius Jasilionis, Jönas, Maria V. and Stefan U.

To David Teze, thank you for having me as your co-author. It was a privilege for me to work with a great researcher like you.

I would like to thank Emma Poaches for her kind help with all the administrative issues, especially for my maternity leave application. To, Frans P. Nilson thanks for your technical support with laboratory equipment and computers.

To Christina, my friend, colleague and officemate, you were always there for me when I needed help. Thank you very much for translating the ‘Popular Summary’.

I appreciate all our discussions, especially the ones that had nothing to do with science! My other office mates Reza and Daniel (we miss you!) thanks, guys for all the laughter and fun memories.

Roya and Andrius, thank you for inviting me to your Tea-party! I enjoyed our discussion about the world politics very much.

Leila A. thank you dear for sharing lab space with me and helping me with the project during my maternity leave. I wish you all the very best.

To the present and past members of the Fermentation group, thank you all for exciting discussions during group meetings, good jokes and fun times.

To Niko (Nikolina) and Maru (Maria), girls you rock! Thanks for being my friend. In my down moments, you both cheered me up and directed me to positivity. I missed those days when we used to stroll around Lund and have Fika.

Now to all the members (past and present) of the Division of Biotechnology, I could not ask for a better and more enjoyable workplace with colleagues like you all, thank you very much.

To the Bangladeshi community and my friends outside KC, thank you for organising all the social activities and allowing me to talk about anything but science!

To my Families, thank you for all the inspirations and I love you all to the moon and back. To my parents, who lives thousands of miles away from me and encouraged me every day, Thank you, mom and dad. To Habiba Rhaman and Kazi Ahmed for your prayers and encouragement. It is always sad and lonely to live abroad without family and friends, but I was lucky to have an extended family here in Sweden, Md. Khuda and Shakila Akhtar. I cannot thank you both enough for your unconditional love and support that you have given to me and my family. To Rumana Sharmin Labony, thank you for your friendship.

Special thanks to my daughter Eiliyah for making me complete. I see a new world through your eyes filled with love and care.

And finally, to my loving husband, Martuza, thank you so much for your love and support, without you I could not have done it. You decided to take care of our daughter instead of pursuing your career further so that I could spend more time in doing my research. My life would be empty without you.



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This picture shows a shallow coastal hot spring located in Reykjanes, Ísafjarðardjúp, NW-Iceland. The thermophilic bacterium *Rhodothermus marinus* was isolated from this very hot-spring. The photo was taken by Ólafur H. Friðjónsson who has been working with this bacterium for a long time. In the picture, the visible cracks are the openings where the hot water flows out. *Rhodothermus marinus* is a marine Gram-negative bacterium that has a beautiful reddish-orange colour. The majority of my thesis work is about enzymes isolated from this bacterium. Our research demonstrates that *Rhodothermus marinus* actually can help us in the production of green bio-catalysts for various industrial processes.



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ISBN: 978-91-7422-730-7  
 ISRN: LUTKDH/TKBT-20/1177-SE

Division of Biotechnology  
 Faculty of Engineering, LTH  
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