# Chemoenzymatic synthesis of anionic alkyl glycosides

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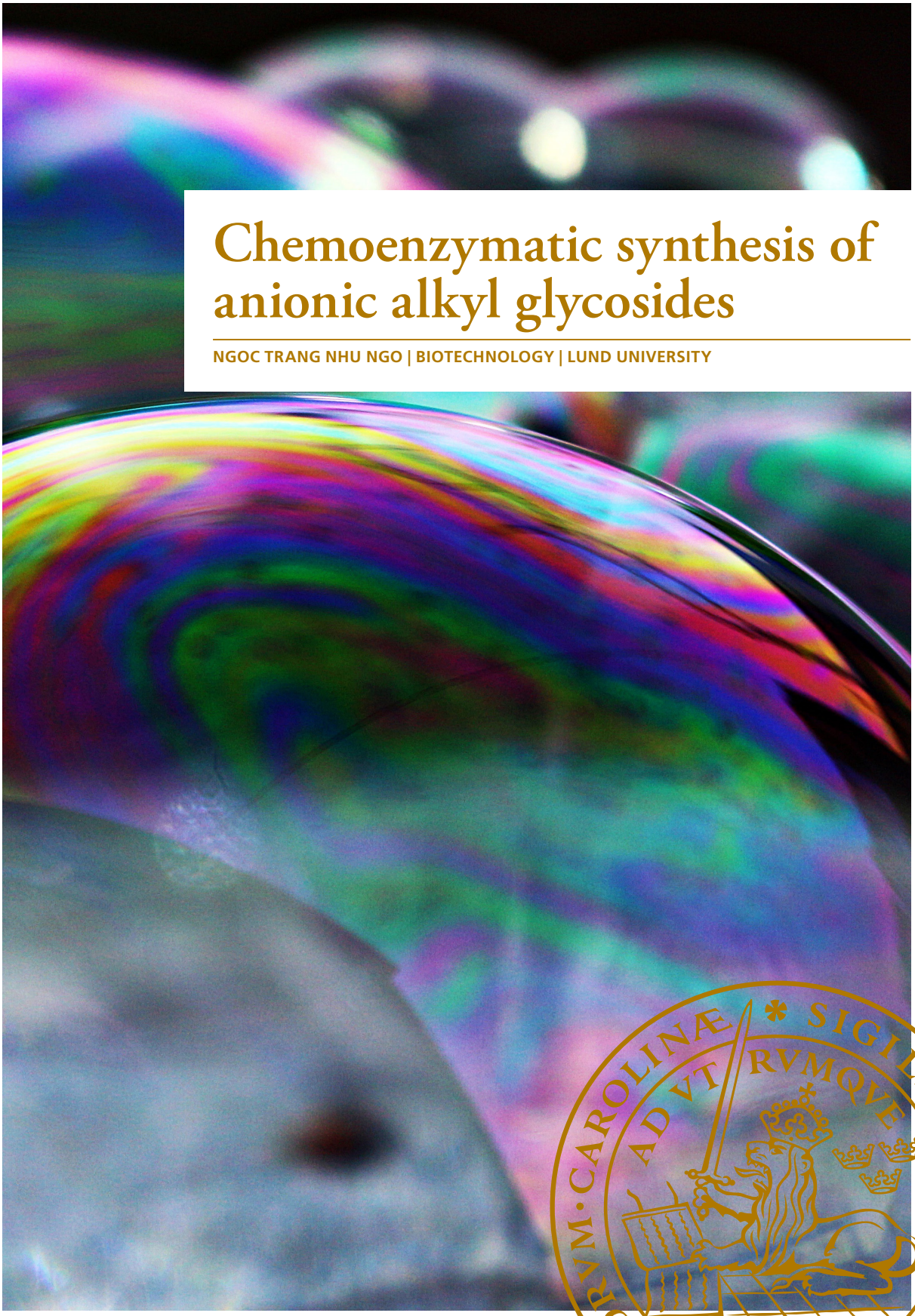

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Ngoc Trang Nhu Ngo


LUND
UNIVERSITY

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Abstract

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In this thesis, the focus is on the synthesis of new derivatives of AGs. The work includes three parts: 1. Production of anionic AGs; 2. Elongation of anionic AGs; 3. Characterization of modified products. In the first part, the method employed was the laccase/TEMPO oxidation of primary alcohol groups of AGs (octyl glucoside – OG, dodecyl maltoside – DDM, hexadecyl maltoside – HDM, dodecyl maltooctaoside – DDMO) to corresponding acids. The influencing factors, including enzyme and TEMPO concentration, were investigated. The reaction showed complete conversion of AGs and high yields at high enzyme and TEMPO concentrations. However, inactivation of laccase by oxidized TEMPO was observed as well as the formation of byproduct ketones. Another disadvantage of the process was the inevitable degradation of glycosides, which however decreases at high TEMPO concentration, except for AGs with long carbohydrate chain, such as DDMO, where the depolymerization became severe. Interestingly, TEMPO can convert any form of AGs, such as monomers, micelles, rod-like and lamellar structures, to give a conversion of 100% without the assistance of co-solvent. The fact that the presence of carboxylic groups in AGs improve the solubility of AGs, especially long alkyl AGs, opens up for their application in aqueous solution. (Paper I-III)

In the second part, the aim was to produce anionic AGs with an oligomeric head group, which is expected to give an improved product with lower cellular toxicity. The extension of the carbohydrate chain was achieved on octyl glucuronic acid (OG-COOH) and dodecyl maltoside diuronic acid (DDM-2COOH), using cyclodextrin glycosyltransferase enzymes (CGTases) as catalyst at pH 5.6. At this pH, these AGs exist mainly in ionized form, which caused a significant decrease in reaction rate as compared to the corresponding underivatized AGs. An increase in pH from 4 to 6 caused about a 25 and 10 times decrease in the initial rates for DDM-2COOH and OG-COOH, respectively. The preference of CGTases for the neutral form over the ionized form of AGs was confirmed by molecular modeling of the enzyme itself and docking experiments with the potential acceptor substrates. A negative charge in the carbohydrate chain could lead to electrostatic repulsion with the negatively charged catalytic base, Asp 356, disturbing the reaction. (Paper III)

In the last part, the interfacial properties of anionic AGs appeared to be highly pH-dependent. Increasing pH caused increased CMC (critical micelle concentration) values of OG-COOH, resulting in low foamability, which is the opposite to the behavior of OG. This can be due to the increased repulsive interactions between negatively charged head groups of OG-COOH, which were formed at elevated pH values. In addition, the size of micelles was studied at different salt concentrations. The results showed that the micellar size increased with an increase in salt concentration. The comparison of the size of micelles formed by OG and OG-COOH indicated that the OG-COOH micellar size is smaller and less influenced by salt and surfactant concentrations than that of OG (Paper I and IV).



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Alkyl glycosides, laccase, TEMPO, oxidation, cyclodextrin glucanotransferases

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*Dedicated to my parents*

# Contents

# Abstract

Growing concerns for environmental issues and product safety for users have shifted research interests towards the use of renewable resources and climate-neutral processes. The enzymatic approach having high selectivity under mild reaction conditions is promising, and the nature-originating products are well accepted. Alkyl glycosides (AGs) consisting of fatty alcohol and sugar are nonionic surfactants applied in many fields, because of their mildness and biodegradability. However, to broaden their applications and replace some of the currently used surfactants, which negatively affect the environment, there is a need to modify the structure of AGs.

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# Popular science

Surface-active substances also called surfactants are defined as amphiphilic molecules being comprised of two distinct parts – water-soluble part (hydrophilic) and water-insoluble part (hydrophobic), that lowers surface tension between liquid-solid or two liquids. Due to the specific structure of the surfactants, they have a wide range of applications such as detergents, personal care products, cosmetic, paint, mineral recovery, wool scouring, pesticide formulations, pharmaceutical preparation and food processing, etc. The annual global market of surfactants accounted about 31 billion dollars with a production of 16 million tons worldwide in 2016. By 2025 the projection for the consumption of the surfactants is about 66 billion dollars. However, hand in hand with increasing demand of the surfactants are environmental and toxicity issues which are attributed to the large accumulation of surfactants in wastewater

Although the surfactants on the surface of wastewater are removed by a combination of several processes (mainly by sorption and aerobic biodegradation) in the wastewater-treatment plants, some of them and their degradation products can still occur in effluents and sewage sludge, which are discharged into surface waters or used as fertilizer in agricultural areas. Some recent literature reported the surfactant occurrence in environmental samples, even in seawater. Surfactants are known to have low human toxicity, but fairly high toxicities toward aquatic organisms. Moreover, some surfactants can be transformed to more toxic degradation products. A particular example of this is nonylphenol ethoxylates. These surfactants can severely irritate the skin and eyes while nonylphenols which are found in the environment as a result of degradation of nonylphenol ethoxylates, is believed to be endocrine disruptors in humans and animals. Worth noting is that the majority of the surfactants are produced from petrochemical raw materials, which is a non-renewable source and facing exhaustion in the future. Therefore, the challenging issue herein is to find alternatives, which can not only be made from renewable sources but also consider environmental compatibility and biodegradation.

Many studies were carried out recently and pointed out the feasibility of utilizing a group of sugar-based surfactants, in which alkyl glycosides (AGs) can be regarded as a promising surfactant of choice for industries. Its consumption represents a world market of 100 000 tons per year and is growing fast. AGs fulfill the desired requirements for a green, non-toxic and biodegradable surfactant class:

1) consist of a fatty alcohol, mainly obtained from natural oil such coconut, palm or rapeseed oil as the hydrophobic part, and sugar, most commonly glucose units from wheat or potato starch, as hydrophilic part.

2) have been found to have a good compatibility with eyes, skin and mucous membranes, reducing irritant effects when combined with anionic surfactants, and are biodegradable under both aerobic and anaerobic condition.

However, AGs have some unexpected disadvantages, comprising of poor solubility in water, particularly for AGs with long alkyl chain, instability at low pH and tendency to aggregate. Moreover, AGs with short hydrophilic chain slightly irritate the skin. Recently, attempts have been made to modify the structure of AGs, mainly on the carbohydrate chain for example: alkylation, acylation, and oxidation to minimize their disadvantages. It is quite tricky to conduct the modification on a specific position chemically, because of the same reactivity of hydroxyl groups on the glucose backbone, requiring many steps with metal catalysts or halide salt. Once again, the environmental concern needs to be addressed, prompting the study of greener alternatives. One promising method is to use enzymatic reactions. With the high specificity, enzymes can perform the reaction in a single step without any harsh chemicals under mild conditions.

Therefore, the aim of this thesis is to search for solutions of the issues above. Firstly, to improve the solubility of AGs, the introduction of carboxylic groups on the glucose skeleton was performed by using laccase/TEMPO oxidation. During the reaction, laccase is first oxidized by $O_2$ and then oxidizes TEMPO to give an oxoammonium ion, known to selectively catalyse the oxidation of primary alcohols. Generally, the method can be considered green and environmentally friendly, since only $O_2$ is required for the regeneration of TEMPO. Carboxylic groups on the glucose units of AGs both improve the solubility of AGs, and transform the nonionic AGs into anionic derivatives at low pH, with potentially interesting properties (Paper I, II and IV).

The second objective was to reduce the irritant effect by extension the hydrophilic chain of oxidized AGs. For this purpose, the elongation of carbohydrate chain was carried out using cyclodextrin glycosyltransferases as catalysts. These enzymes have a tendency to link glucose or cyclodextrin as donor to the carbohydrate chain of oxidized AGs as acceptor. Compared to chemical reactions, this enzymatic method gives good yields under mild conditions without protection/deprotection steps and chemical wastes (Paper 3). These results bring us closer to the goal of the production of a new renewable generation of surfactants by eco-friendly methods.

# List of Paper

This doctoral thesis is based on the following papers, referred to by their Roman numberals

I. Ngoc T. N. Ngo, Carl Grey, Patrick Adlercreutz. (2019) Chemoenzymatic synthesis of the pH responsive surfactant octyl β-D-glucopyranoside uronic acid. Appl Microbiol Biotechnol. 104: 1055-1062.

II. Ngoc T. N. Ngo, Carl Grey, Patrick Adlercreutz (2020) Efficient laccase/TEMPO oxidation of alkyl glycosides: The effects of carbohydrate group and alkyl chain length (Manuscript).

III. Ngoc T. N. Ngo, Javier Linares-Pastén, Carl Grey, Patrick Adlercreutz (2020) Novel oligomeric anionic alkyl glycosides by two alternative routes using either laccase/TEMPO oxidation or cyclodextrin glucanotransferase (CGTase)-catalyzed transglycosylation. (Manuscript).

IV. Ngoc T. N. Ngo, Nikolina Barchan, Carl Grey, Patrick Adlercreutz (2020) Surface properties of carboxylated alkyl glycosides (Manuscript).

# My contribution to the papers

All work described in this thesis was performed under the supervision of Prof. Patrick Adlercreutz, Assoc. Prof. Carl Grey at the Division of Biotechnology.

I. I planned the work together with my coauthors. I carried out the experimental work and wrote the manuscript with help of Patrick Adlercreutz, I revised the manuscript together with Carl Grey and Patrick Adlercreutz.

II. I planned the work together with my coauthors. I carried out the experimental work and wrote the manuscript with help of coauthors.

III. I planned the work together with my coauthors. With the exception of molecular modelling, I carried out all experimental work. The first draft was written by Patrick Adlercreutz.

IV. I planned the work together with my coauthos. I measured the surface tension and determined CMC values with varying pH. I did DLS measurement with help of Nikoline Barchan

# 1. Introduction

The development of modern technology brings mankind high living quality, but severely affects the environment with consequences such as global warming, increasing release of hazardous chemicals and heavy metals, and shortage of natural resources. These reasons have promptly made scientists change strategies toward alternative materials and technology, which are sustainable and more environmentally friendly. Among them is biotechnology, particularly biocatalysis using microorganisms and enzymes instead of chemical catalysts for synthetic purposes, to minimize the use of toxic chemicals and waste, and environmental impact. These concepts follow the "12 principles of Green Chemistry" presented by Paul Anastas and John Warner, which is the standard for current research with respect to the design of environmentally friendly products and synthetic processes (Anatas and Warner 1998). In principle, a biocatalytic process is conducted under mild reaction conditions, such as room temperature, in aqueous media, neutral pH, and generates minimum amount of toxic waste. Other advantages of this methodology are the reaction selectivity of enzyme or whole-cell system and good reaction efficiency (Woodley 2008; Wohlgemuth 2010).

Moreover, the production of biodegradable products has attracted considerable attention. Surfactants are among the abundantly used chemicals in various fields in everyday life. However, some of them are formed from non-renewable resources, and some cause negative effects on aqueous environments. AGs, non-ionic surfactants, are known as natural surfactants because of being generated from fatty alcohols and sugars. They are considered to be very mild on skin and completely biodegradable. Thus, they are regarded as among the best alternatives in the world of surfactants. However, to be able to widely apply them in many fields, AGs must be modified. It is difficult to modify the structure of AGs using chemical methods, because of the similar reactivity of hydroxyl groups on the carbohydrates of AGs. The general ways to modify carbohydrates are the use of protection/deprotection steps on the hydroxyl groups or use the halide-catalysts, both of which release toxic chemical waste. Thus, the current research focuses on replacing the chemical processes with enzymatic methods, which give high reaction selectivity under mild conditions.

In this thesis, attempts have been made concerning the synthesis of carboxylated AGs with oligomeric head groups, through biocatalytic routes employing laccase and CGTases. The presence of carboxylic groups in AGs can enhance the solubility of long alkyl chain AGs and give negative charges, even at low pH values. They are expected to act as anionic surfactants, which are insoluble in acidic medium.

## 1.1. Scope of the thesis

The aim of the thesis is to develop enzymatic synthesis of novel derivatives of AGs that can be used to supplement and replace commercially available surfactants. The thesis is based on four manuscripts, of which one is published. The contents are as follows:

Paper I-III investigate the reaction conditions for selective oxidation of primary alcohols of AGs and elongation of the carbohydrate chain of AGs and anionic AGs. In the first approach, carboxylated derivatives of AGs are synthesized using the laccase/TEMPO system. The influencing factors such as temperature, $O_2$, the concentration of TEMPO, laccase and AGs were investigated to furnish high yields. The papers describe the process of product formation. In the second approach, the reaction takes place with the catalysis of CGTases from *Bacillus macerans* (Amano) and *Thermoanaerobacter* sp. ATCC 53627 (Toruzyme® 3.0L). Anionic acceptors utilized were carboxylated AGs purified from the first approach. The focus was on the influence of pH on the acceptor specificity of CGTases and reaction rate.

Paper I and IV study the surface properties of anionic AGs by measuring surface tension, as well as determining CMC at different pH values. A comparison of the effect of salt concentration on AGs and anionic AGs was done through measurements of the size of micelles.

# 2. Surfactant

## 2.1. Properties

Surface active molecules or so-called surfactants are amphiphilic molecules, consisting of a polar head and a hydrophobic tail. The hydrophobic part is usually a hydrocarbon chain of varying length. The difference of the nature of head groups is a feature distinguishing groups of surfactants (anionic, cationic, nonionic, and zwitterionic surfactants) (Fig. 2.1). One of the most widely used ionic surfactant is sodium dodecyl sulfate, while the most famous nonionic ones are based on poly(ethylene oxide). Recently, AGs appear to be an environmentally promising nonoionic alternative.

Surfactants can adsorb effectively on liquid-air and water-oil interfaces, decreasing surface and interfacial tension. In addition, surfactants have a remarkable ability to self-assemble in aqueous solution (e.g. micelles). The structure formed can reduce the exposure of hydrophobic chains, and trap fatty or organic materials in water. This is a fundamental property of surfactants in the cleaning action of detergents.

### 2.1.1. Micelles and critical micelle concentration

It is found that when the concentration of the surfactant reaches a certain level, all properties change suddenly. This concentration is called the critical micelle concentration (CMC). Hamley (2000) presented a relationship between the concentration of surfactant and physicochemical properties of the surfactant solution such as osmotic pressure, electrical conductivity, surface tension, etc. Above the CMC, surfactants aggregate to form liquid-like often near spherical structures, called micelles. The formation of micelles (micellization) is an alternative to the adsorption mechanism for minimizing the system's energy when all the surfaces are saturated with surfactant.

The structure of micelles is dependent on the relationship between the tail chain volume ($V_{surf}$), the critical tail chain length ($l_c$), and the head group area at the head-tail interface ($\alpha_0$). All of the factors are defined in the following equation.

$$\mathrm{CPP} = \frac{V_{surf}}{l_c * \alpha_0}$$

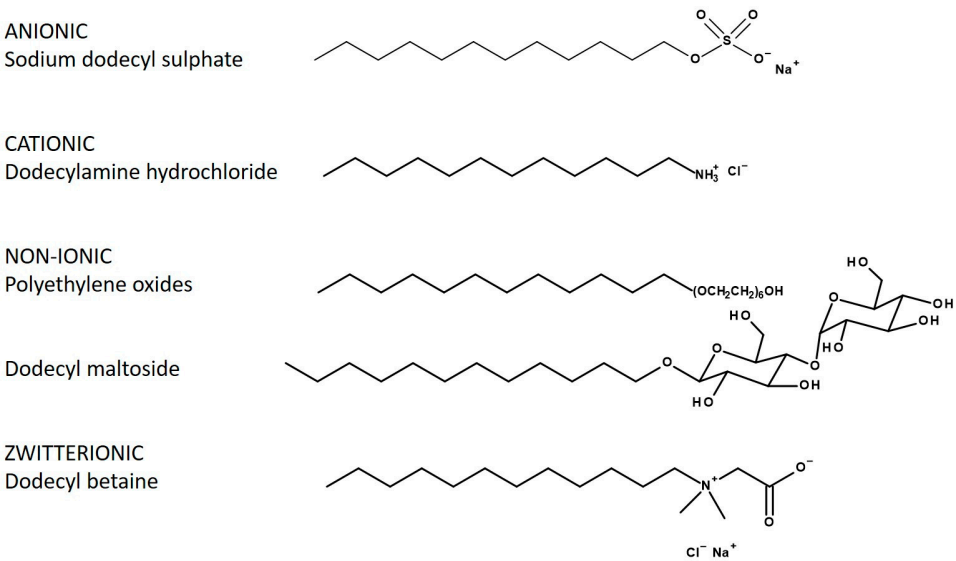


**Fig. 2.1.** A list of typical surfactant molecules

Critical packing parameter (CPP) is a geometric parameter of the surfactant, and connected directly to the structure of the micelles. If, for example, a surfactant has a single chain, and the alkyl chain volume is small, the overall shape of the surfactant is more similar to a cone and CPP is much lower than 1. In this case, aggregates like spherical micelles or cylindrical micelles are favorable. When CPP is close to 1, corresponding to surfactants having two hydrophobic chains and small head groups areas, a bilayer is favored (Fig. 2.2).

Moreover, the shape of surfactant structures is also affected by their concentration. In the case of micellar surfactants, at elevated concentrations spherical micelles can aggregate to form rod-like micelles like hexagonal structures (Fig. 2.2).

In each micelle, there are often many surfactant molecules, about 50-100 for spherical micelles, or higher for other structures. This number is called the aggregation number and is defined by the ratio of the volume of the micelle to the volume of a surfactant molecule. This value increases with decreasing CMC and with increasing salt concentration.

Salts do not influence equally the CMC for non-ionic and ionic surfactants. For non-ionics, no significant influence is observed. For ionics, there is a reduction of the repulsion force between the charged head groups; because of this, the CMC value decreases with increasing salt concentration. Detailed information about micelles can be found in textbooks such as "Introduction to Soft Matter" by Ian W. Hamley and "Introduction to applied colloid and surface chemistry" by Georgios M. Kontogeorgis and Soren Kiil.

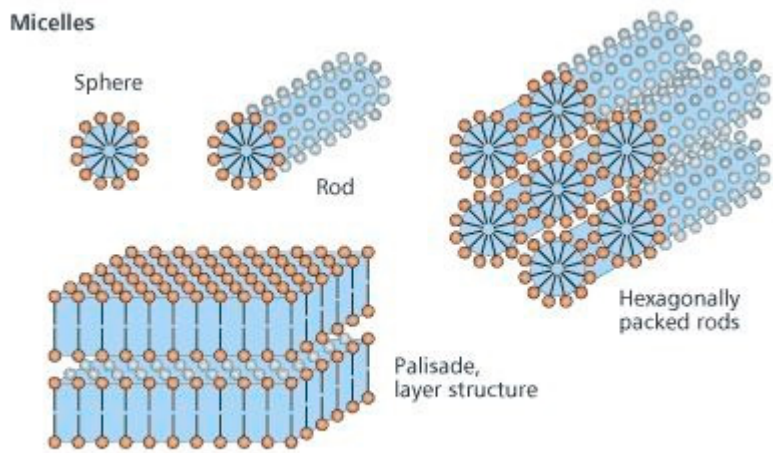


**Fig. 2.2.** Aggregate structures and arrangements of surfactant molecules in aqueous solution (Adopted from the website: www.sci-toys.com).

### 2.1.2. Anionic surfactants and their foamability

Surfactants are a broad range of chemicals that play a crucial role in many fields. Among classes of surfactants, anionic surfactants dominate the world surfactant market, with 60% of the world production. Anionic surfactants are characterized by negatively charged hydrophilic polar groups, of which carboxylic acid, sulfonic acid, and phosphoric acid are typical groups used. They represent the most important group of surfactants used in cleaning products because of their foaming abilities. Theoretically, the anionics leads to a surface charge of the foam, resulting in an electrostatic repulsion at the surface. Thus, the stability of a foam is promoted by maintaining the thickness of the liquid films. On the other hand, if the surfactants become ionized completely, the repulsion force between similarly charged

molecules in the monolayer can increase and lead to a weak and unstable film (Adam 1921). Kanicky *et al.* (2000) investigated the stable foam formed by sodium laurate at various pH values. It was concluded that improved foamability and foam stability were observed to occur at or very near the bulk pKa of sodium laurate. This was ascribed to the intermolecular interaction between ionized to unionized soap molecules at the surface. The interaction reduces the head areas of surfactant molecules, which is supposed to be a factor lowering surface tension. Also, the shorter intermolecular distance observed narrows the pores through which water molecules can evaporate, leading to foam drainage.

Moreover, the foam stability is also influenced by the layer of surfactants around a bubble, which prevents aggregation of the foam, and thus, the collapse of the foam system. Between foams are lamellar films that are formed by surfactant micelles and become the building blocks of the foam system. As mentioned above, at high concentrations of surfactant, the aggregation of micelles leads to the formation of hexagonal or lamellar structure. Lee *et al.* (2014) showed that the higher foamability and foam stability created from sodium dodecyl sulfate (SDS) are obtained at SDS concentrations above its CMC value.

## 2.2. Alkyl glycosides

Today, when ecological concern and product safety for users, particularly concerning the skin-irritation effect, is growing, the demand for new surfactants that are more environmentally friendly and milder to the skin is of significant interest. AGs, non-ionic surfactant, appear to be a potential candidate because of their biodegradability, compatibility with the skin, and efficiency as surfactant. Also, as compared with Triton X-100, AGs are better, due to higher chemical stability in alkaline solution and superior protein extraction capacity (Lin *et al.* 1979; Svensson *et al.* 2009a). However, so far, these AGs find use mainly in detergent and personal care products where anionic surfactants hold a dominant position. Therefore, the derivatization of AGs is currently being pursed to modify the physicochemical properties of AGs. The modification of AGs will be discussed below.

### 2.2.1. Modification of AGs

The modifications of AGs mainly occur on the carbohydrate chain, for example methylation, esterification, transformation of nonionic into their ionic counterparts, by condensation of AGs with citric acid/tartaric acid/maleic anhydride/ sodium hydroxypropyl sulfonate. However, among a range of modified products, anionic

derivatives exhibit different properties compared to the original AGs, and as a result, they are used in the formulations of personal care products (Hill 2010; Seweryn *et al.* 2019; Konya *et al.* 2004). This prompt studies on the formation of a variety of anionic AGs. One route for the introduction of negative charges is the selective oxidation of the primary alcohols of AGs to the corresponding acids. Generally, owing to the similar reactivity, the hydroxyl groups of the carbohydrate must undergo protection/deprotection steps before the chemical oxidation starts. Alternatively, using metallic catalysts carrying ligands can cause steric effects, resulting in that the oxidation only takes place on primary alcohol groups of the carbohydrate. Recently, an enzymatic method involving a laccase enzyme and TEMPO mediator was reported. TEMPO is known to selectively catalyse the conversion of primary alcohols to acids, while the laccase acts as a battery, storing electrons from the substrate oxidation to reduce oxygen molecules. So the whole reaction is the combination of the alcohol oxidation and the reduction of oxygen to produce water. The reaction mechanism and the influencing factors will be discussed in detail in chapter 3, 4 and 5 and Paper I-II.

Another modification of AGs is the elongation of the carbohydrate chain of AGs, through transglycosylation by CGTase enzymes. It is of considerable interest since according to a previous report about nonionic surfactants, the elongation of hydrophilic chain lowers the cellular toxicity of the surfactant. (Ekelund et al. 2005). The elongation process occurs through a transfer of sugar residues from a donor molecule α-cyclodextrin (α-CD) to an acceptor molecule (AGs), following the retaining double displacement mechanism. Svensson and co-workers (2009a and 2009b) described acceptor and donor specificity, and suitable reaction conditions for the synthesis of AGs with oligomeric head groups. Furthermore, chapter 6 and Paper III report the employment of new acceptors - carboxylated derivatives of AGs - in the transglycosylation, in which the presence of negative charges on the acceptor has a significant influence on the CGTase enzyme, and thus on the overall reaction rate.

### 2.2.2. Applications

Owing to attractive physico-chemical properties, AGs show interesting effects for many applications. Unlike other nonionic surfactants, AGs exhibit synergistic interactions with various primary surfactant systems, which make the formulations of products more effective to achieve a reduction of surfactant content without affecting the performance level (Von Rybinski and Hill 1998). In addition to good foamability and mildness to the skin, AGs have efficient cleaning performance, which is not affected by pH. For these reasons, AGs are found in the ingredient list of a majority of detergents in personal care cleaning products.

The use of AGs in microemulsions has been studied intensively. Microemulsions are formed by a system consisting of water/oil/surfactant and used as a vehicle for drug delivery. Microemulsions stabilized by ethoxylated nonionic surfactant are very susceptible to temperature influences, which is the basis of the phase inversion temperature phenomenon (PIT) (Von Rybinski and Hill 1998). PIT is a phenomenon in which an oil/water emulsion inverts into a water/oil emulsion at high temperature and vice versa. Alternatively, AGs with their characteristic phase behavior, which is less affected by temperature, gives a temperature independent microemulsion at the right AGs/co-surfactant ratio (Pantelic and Cuckovic 2014). Moreover, due to their capacity to preserve protein activity and conformation in solution, AGs find use for the solubilization of membrane proteins. For example, AGs are used for binding to bacteriohodopsin which is a membrane protein found in the purple membrane of *Halobacterium salinarum* (Santonicola *et al.* 2008).

# 3. Laccase enzyme

## 3.1. Introduction to laccases

Laccase (benzenediol: oxygen oxidoreductases: EC 1.10.3.2) is a glycosylated four-copper containing oxidase, which belongs to the small family of blue multi-copper oxidases (Thurston 1994). A Laccase was discovered in 1883 by Yoshida (Yoshida 1883), and characterized as a metal containing oxidase by Bertrand in 1985 (Bertrand 1985). Thus, laccases are regarded as one of the oldest known enzymes. They are mainly found in plants and fungi (Harvey and Walker 1999; Mayer and Harel 1979; Solomon *et al.* 1996). However, the purification of plant laccases is challenging because the laccases may be bound to cell walls in some higher plants, making their detection difficult in crude extracts, which contain a variety of oxidative enzymes. The application of plant laccases is therefore far more limited than fungal laccase.

Laccases can catalyse one-electron oxidation of substrates coupled with the simultaneous four-electron reduction of molecular $O_2$ to $H_2O$. The use of $O_2$ makes laccases particularly attractive as biocatalysts from the environmental point of view. Substrates oxidized by laccases include polyphenols, diamines, methoxy-substituted phenols and some inorganic compounds. The action of laccases on non-phenolic substrates may be expanded by use of so-called mediators. Mediators of natural origin are already found in biomass, while synthetic mediators include various types of chemical compounds such as TEMPO (2,2,6,6-tetramethyl-piperidine-1-oxyl radical) and its derivatives, ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), HBT (hydroxybenzotriazole), and HPI (N-hydroxyphthalimide) (Morozova *et al.* 2007; Christopher *et al.* 2014). A broad range of substrates which are converted by laccases are associated with the wide choice of mediators, resulting in the diversity of applications of laccases.

Laccases are at present used for some industrial-technical applications in the areas food, organic synthesis, medicinal and personal care (Feng 2005). Two examples are the treatment of dye waste streams and the bleaching of indigo dye (Xu 1999). Laccases were also used commercially for preparing cork stoppers for wine bottles. Its function is to reduce the characteristic cork taint which is frequently imparted to the bottled wine (Conrad *et al.* 2000). The use of laccase has spread to the field of fossil fuels. A study reported that the laccase-catalysed oxidation of inorganic and

organic sulfur compounds facilitates the elimination of these compounds from solid coal particles (Villaasenor *et al.* 2004).

## 3.2. Structure of laccase

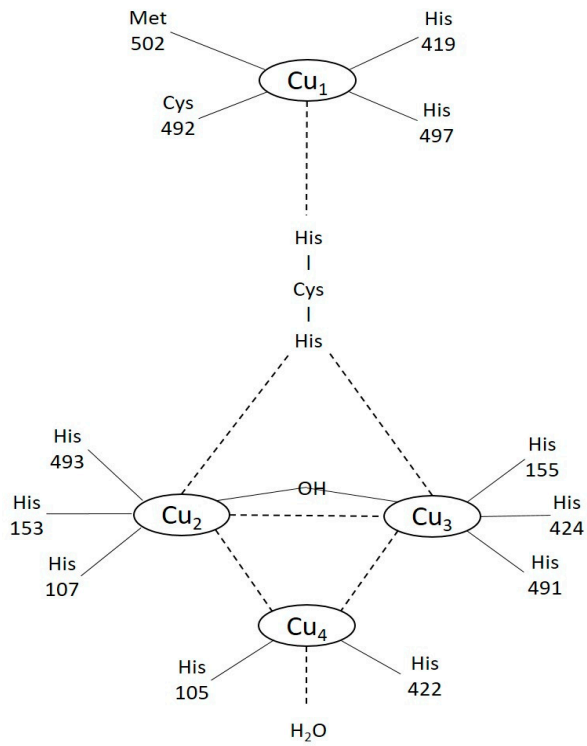


**Fig. 3.1.** Copper centers of the laccase (CotA) from *Bacillus subtilis*. Four Cu molecules represent three types: $Cu_1$ is for T1 Cu, $Cu_2$ and $Cu_3$ is for T3 Cu , $Cu_4$ is for T2 Cu.

Until now, more than 100 laccases have been purified from fungi and characterized to various extents. The molecular mass of the monomer ranges from about 50 to 100 kDa with acidic isoelectric points around pH 4.0. They are glycoproteins with carbohydrate portions of 10-30 % of the molecules for fungal laccases and up to 45 % for plant laccases (Baldrian 2006). The carbohydrate portion is supposed to

contribute to the high stability of the enzymes and protect them from proteolysis and inactivation by radicals (Yoshitake *et al.* 1993; Ko *et al.* 2001).

The laccase molecule usually contains four copper atoms located at three redox sites (Type 1, Type 2 and Type 3 Cu pair) (Fig. 3.1). Three types of copper can be distinguished by their spectroscopic and electron paramagnetic resonance (EPR) parameters (Malmström 1982; Solomon *et al.* 1996). The T1 center is responsible for the blue color of the enzyme solution at an absorbance of 610 nm and is EPR detectable. The T2 Cu, non-blue, is also EPR detectable. The T3 Cu is comprised of two Cu atoms in a binuclear conformation. It lacks an EPR signal, but displays a weak absorbance around 330 nm (Solomon et al. 1996; Quintanar *et al.* 2005).

The T1 Cu is coordinated by three ligands including two conserved histidines (His) and one cysteine (Cys) as equatorial ligands and axial ligands of variable nature, usually methionine in bacterial and leucine or phenylalanine in fungal laccases. These ligands form a trigonal structure. The redox potential of the Type 1 varies from 430 mV to 780 mV with different laccase types (Xu *et al.* 1996; Gianfreda *et al.* 1999). It is revealed that the higher the potential of the T1 Cu, the higher the catalytic efficiency (Xu *et al.* 1996). With laccases having high redox potential of the T 1 Cu, the T 1 Cu site is the place where the substrate oxidation takes place.

The T2 Cu and T3 Cu are close together and form a trinuclear cluster, where reduction of molecular $O_2$ and release of $H_2O$ take place (Spira-Solomon *et al.* 1986; Cole *et al.* 1990). The T2 Cu binds two His and each Cu of T3 Cu binds three His (Solomon *et al.* 1996; Messerschmidt and Huber 1990). The latter two Cu are bridged by a hydroxyl group (Claus 2004). Besides, laccase contains a conserved His-Cys-His tripeptide sequence which is located between the T1 and T2/T3 Cu sites and might be important in mediating electron transfer (Piscitelli *et al*. 2010).

## 3.3. Catalytic mechanism

Generally, during the oxidation process, the copper centers of laccase drive electrons from a reducing substrate to molecular $O_2$ to form $H_2O$ via three main steps (Fig. 3.2). Firstly, the oxidation of substrate takes place at the T1 Cu site. One electron from the donor substrate is transferred into the T1 Cu ($Cu^{2+} \rightarrow Cu^{1+}$) through a direct interaction with a His in the T1 Cu site. The electron, then, is transferred internally from the T1 Cu site to a trinuclear cluster T2/T3 Cu site through a brigde of a His-Cys-His sequence. At that moment, $O_2$ molecules diffuse into the cluster through the solvent accessible entry channel of the enzyme. At the trinuclear cluster, the reduction process of $O_2$ involves $O_2$ cleavage and a transfer of $H^+$ from a carboxylic Glu residue. As a result, the first $H_2O$ molecule is produced

and diffuses out of the trinuclear cluster site through the solvent accessible exit channel (Kunamneni *et al.* 2008; Arregui *et al.* 2019).

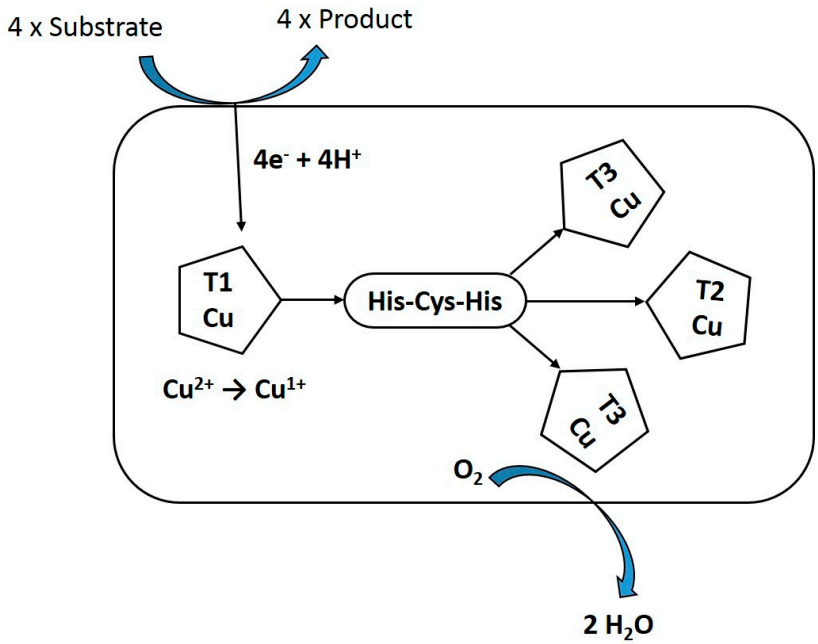


**Fig. 3.2.** General scheme of the catalytic mechanism of laccase

## 3.4. Laccase-mediator system

The mediated system in the presence of laccase has been extensively discussed previously. The role of the mediator is to enhance the catalytic activity of laccase, with respect to substrates which are too large to penetrate into the enzyme active site or have a particularly high redox potential (Kunamneni *et al.* 2008). Employed mediators must have stable oxidized and reduced forms and meet the requirement of multiple reaction cycles in the redox process (Johannes and Majcherczyk 2000). The activity of the mediators is claimed to follow the pathway: The mediator acts as electron-transfer agent between laccase and substrate, where its oxidized form can diffuse away from the catalytic pocket of the enzyme due to its limited size, and is capable of oxidizing substrates (Banci *et al.* 1999). Thus, because the actual oxidation of substrate occurs outside the enzyme, possible steric and polar inhibiting interactions with the laccase are eliminated.

# 4. Selective chemical oxidation of carbohydrates

The selective oxidation of the primary alcohol groups of carbohydrates is one of the important available carbohydrate modifications, since it yields not only polyelectrolytes but also valuable intermediates. Besides, the use of oxidized carbohydrates has been investigated in a number of applications. Oxidized celluloses are, for example, widely used in medical applications such as absorbable hemostatic scaffolding materials (Dias and Peplow 2003), postsurgical adhesion prevention layer (Wiseman *et al*. 2002), as carrier material for agricultural and cosmetic products (Banker and Kumar 1995). In the food sector, oxidized starches are often used in batters and breadings for coating in a wide variety of foodstuffs, since they provide good adhesion and a crispy texture after frying (Thomas and Atwell 1997).

Because the hydroxyl groups on carbohydrates have nearly the same reactivity, selective oxidation of the primary alcohol becomes quite tricky with traditional chemistry. Attempts have been made to reduce regio and chemoselectivity problems, which often makes it necessary to protect functionalities not involved in the desired transformation. In the following chapter, oxidation of primary alcohols to carboxylic groups by using metal catalysts as well as mediated systems will be discussed.

## 4.1. Metal catalysts

Two metal catalyst systems seem to have good potential in the selective oxidation of carbohydrates, comprising of heterogeneous and homogeneous catalysts. However, in the oxidation of water-insoluble polymer substrates like starch or cellulose, heterogeneous catalysts cannot be applied. In contrast, in the oxidation with soluble substrates, these catalysts facilitate the separation of products after the reaction by filtration, allowing catalyst recycling. Meanwhile, the oxidation of insoluble and soluble substrates can be catalysed by homogeneous catalysts. However, the reuse of the catalysts is challenging (Vinke *et al*. 1992).

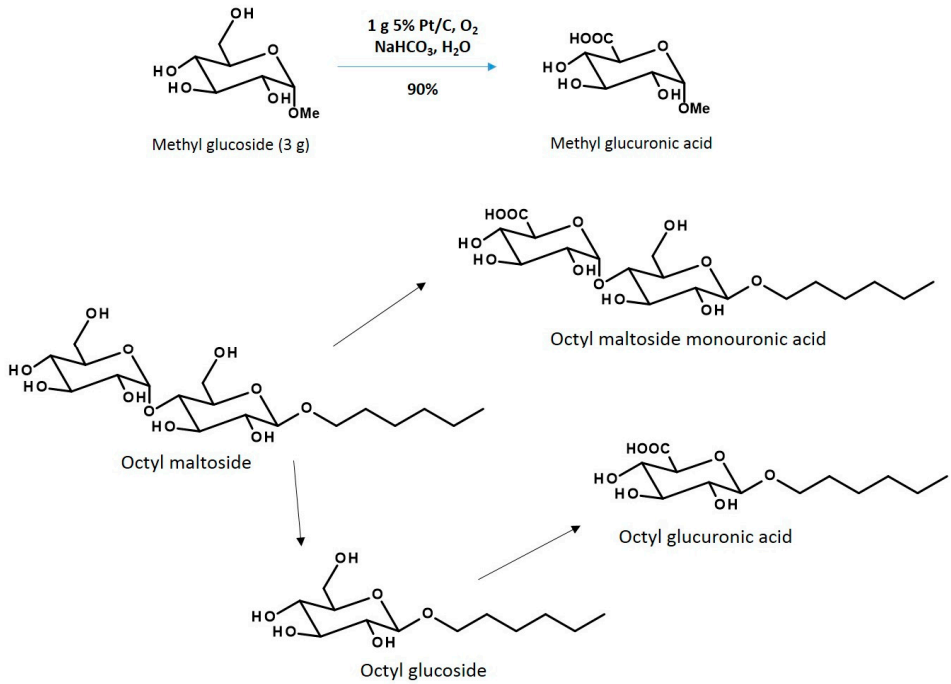


**Fig. 4.1.** Scheme of the oxidation of methyl glucoside (5% Pt/C) and octyl maltoside (5% $Pt/Al_2O_3$)

### *Heterogeneous catalysts*

When using heterogeneous catalysts in the oxidation, soluble substrates like carbohydrates with short length or their derivatives such as AGs are required. The catalyst system, which seems to have good potential in selective oxidation of carbohydrate involves platinum-catalysed oxidation with $O_2$ in water. The subtrates did not undergo any protection processes before being oxidized. Another advantage of this catalyst system is the easy removal of the metal from the reaction mixture by filtration and no other inorganic salts are needed. The reagent mostly oxidizes hydroxyl groups at $C_1$ and $C_6$ to give an aldaric acid. Also, the hemiacetal group at $C_1$ is preferentially oxidized, but when it is blocked as in alkyl glucosides, the primary hydroxyl group will undergo oxidation with very high selectivity over the secondary hydroxyl groups (Madsen 2001) (Fig. 4.1). However, the platinium catalysed oxidation has been disappointing when applied to alkyl maltoside. During the reaction, the primary hydroxyl group at $C_6$ of the terminal glucose was oxidized exclusively. This is assumed due to the oxidation of alkyl maltoside in a micellar structure where the accession of Pt to $C_6$ in the second glucose unit is easier than

that to $C_6$ in the first glucose unit. Some byproducts from the oxidative splitting were obtained, including alkyl glucoside and alkyl glucuronic acid (Vinke *et al*. 1992) (Fig. 4.1). The drawback of the reaction is that a large amount of platinum catalyst is required, as is evident in the oxidation of glucose and fructose. Oxidizing 3 g glucose to give 90% glucuronic acid required 1 g Platinum, while 0.5 g of Platinum was used for oxidation of 0.5 g of fructose. Worth noting is that the catalyst is rather sensitive to $O_2$, and will be poisoned if the $O_2$ concentration in the liquid phase is too high (Madsen 2001).

### *Homogeneous catalysts*

The oxidation of primary hydroxyl groups in carbohydrates generally uses strong oxidizing agents to give carboxylic acid. The trade-off is that the substrates in the reaction must undergo protection/deprotection steps to prevent the undesired secondary alcohol oxidation.

Of the catalyst systems, the John oxidation (chromium (VI) oxide, sulfuric acid) is an often-applied protocol for preparation of uronic acid even on large scale. However, the method usually requires a large excess of oxidizing reagent to approach a good yield. Particularly, Van Boeckel *et al*. (1985) reported the oxidation of allyl 2,3,4-tri-O-phenylmethyl-$\alpha$-D-glucoside using the John oxidation with 2 eq. of reagent to obtain 77% of the corresponding uronic acid. Another drawback is that the highly acidic reaction condition usually causes, to some extent, a cleavage of protecting groups such as acetals and sulfides during the oxidation (Madsen 2001).

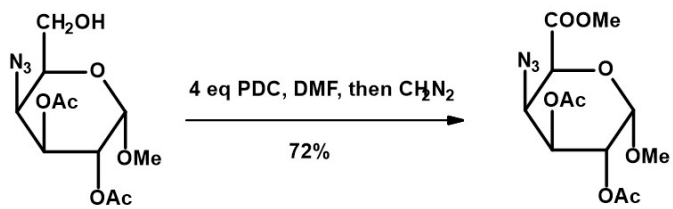


**Fig. 4.2.** Scheme of the oxidation of glucose using PDC as catalyst.

An alternative chromium (VI) based oxidant for preparation of uronic acids is pyridinium dichromate (PDC). Like John oxidation, the use of larger excess of the reagent (4 eq. or more) was also required to reach complete oxidation. The method used an aprotic solvent like DMF or dichloromethane, thus preventing the cleavage of protecting groups such as acetals and sulfides (Madsen 2001) (Fig. 4.2).

Oxidizing selectively unprotected or partly protected glycosides is generally not possible using the above methods, but feasible if using milder and more selective oxidants, which take advantage of the steric hindrance among hydroxyl groups of carbohydrates. Such a catalyst system was investigated by the Boelrijk group, using ruthenium polypyridyl complexes as catalyst in the presence of $NaBrO_3$. The function of polypyridyl ligands is to protect the catalyst from product inhibition and create the steric effects, resulting in substrate selectivity of transition metal centers (Boelrijk *et al*. 1995 and 1996). Worth noting is that the method required only a tiny amount of metal catalyst for complete oxidation. Particularly, Boelrijk carried out the oxidation of octyl glucoside (OG) and decyl maltoside catalysed selectively by $[Ru(2\text{-}(phenylazo)pyridine)_2(H_2O)_2]^{2+}$ to give uronic acids without protection/deprotection step. The presence of dicarboxylic acid in oxidation of decyl maltoside was detected, which was not found as using Platinium catalysts. The major reaction in the oxidations, however, was the hydrolysis reaction cleaving the $\alpha$-1,4-O link between the alkyl chain and the carbohydrate moiety (Boelrijk *et al*. 1996).

## 4.2. TEMPO-mediated system

### 4.2.1. Reaction mechanism

TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl) is a secondary amine nitrogen oxide in which an unpaired electron is delocalized between the N and O atoms. Stable radical TEMPO is a well-known catalyst in the selective oxidation of primary alcohols in the presence of secondary ones. Its applications in polysaccharide oxidation showed a regioselective conversion of $C_6$ primary hydroxyls to carboxylic groups. In general, TEMPO in combination with single $O_2$ donors (NaClO, NaBrO) or in combination with molecular $O_2$ and transition metals (Cu or laccase enzyme including Cu) efficiently oxidize the primary alcohol of carbohydrates without protection/deprotection steps (Sheldon and Arends 2004; Pierre *et al*. 2017).

***Mechanism of the oxidation***

Firstly, TEMPO undergoes a one-electron oxidation by the first oxidant to form the oxoammonium ion ($TEMPO^+$), which is a strong oxidizing agent. Then, a primary hydroxyl group of the substrate act as a nucleophile and attacks $TEMPO^+$ to give an adduct, which subsequently decomposes to the substrate aldehyde and hydroxylamine. The comproportionation reaction between $TEMPO^+$ and

hydroxylamine results in the regeneration of two TEMPO molecules and a proton, thus completing the catalytic cycle (Fig. 4.3.) (Tromp *et al.* 2010, Bragd *et al.* 2001).

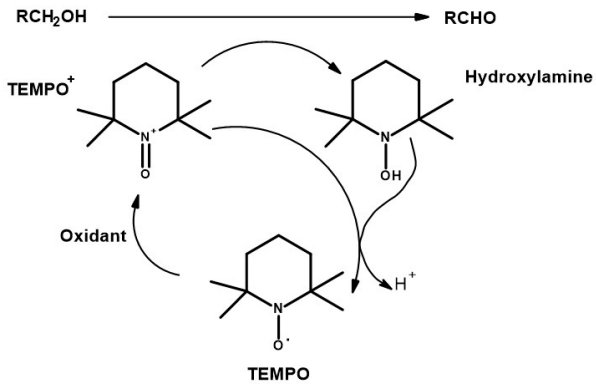


**Fig. 4.3.**Reaction mechanism of TEMPO-mediated oxidation of primary alcohols.

The aldehyde, in the presence of water, equilibrates with the corresponding hydrate that can be oxidized via a similar mechanism to the corresponding acid. Thus, if the water solubility of the aldehyde is too low or its hydration equilibrium is unfavorable, the reaction could stop at the aldehyde level. It is worth noting that the subsequent oxidation of aldehydes to carboxylic acids may be affected by the presence of the first oxidant, if it is applied in excess (NaClO or $O_2$).

The TEMPO-mediated oxidation of primary alcohols can be performed in basic or acidic conditions. The adduct of $TEMPO^+$ and alcohol is formed via a linear transition state under acidic conditions or the five-membered transition under basic conditions. The latter transition state is more compact, leading to quicker oxidation and a greater selectivity for primary alcohol versus secondary ones (Fig. 4.4) (Tojo *et al.* 2007*)*.

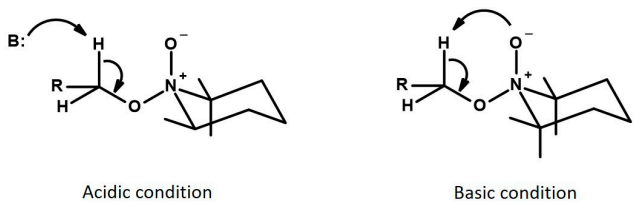


**Fig. 4.4.**. Transition state of the adduct of $TEMPO^+$ and alcohol in basic and acidic conditions

### 4.2.2. TEMPO/NaOCl/NaBr

The TEMPO/NaClO/NaBr system was first applied to partially protected glycosidic carbohydrates, i.e. methyl glucoside and OG by Davis and Flitsch (1993) to give the corresponding uronic acids with good yields. This report paved the way for numerous studies on polysaccharide oxidation using this system (de Nooy *et al.* 1995 and 1996; Cosenza *et al.* 2015; Pönni *et al.* 2014).

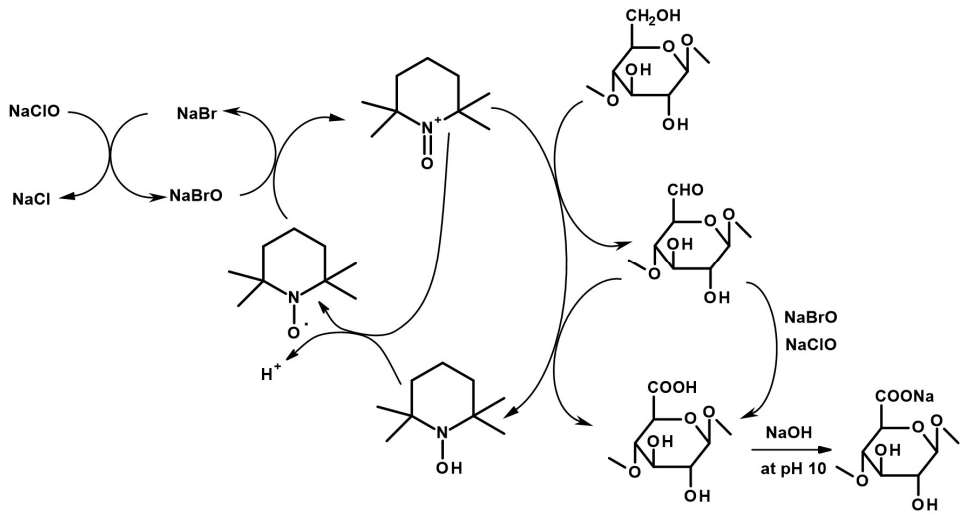


**Fig. 4.5.** Catalytic oxidation mechanism of C6-OH groups of cellulose by TEMPO/NaBr/NaClO in water at pH 10.

The TEMPO/NaClO/NaBr oxidation of polysaccharides is described according to the mechanism shown in Fig. 4.5. The $TEMPO^+$ is generated by oxidation of TEMPO with NaOCl as a first oxidant at low temperature (0-4 °C) and under basic conditions (pH 9-12). The oxidation process can be monitored from the pattern of aqueous NaOH consumption, which is continuously added to the reaction mixture to maintain the pH during the oxidation. Primary alcohols under alkaline medium, form an adduct with $TEMPO^+$ via the five-membered transition state. The intermediate is expected to be formed easily at high pH, due to loss of a proton, which could explain the increase in reaction rate. De Nooy *et al.* (1995) investigated the influence of pH on the oxidation of methyl α-D-glucopyranoside and observed a dramatic increase in oxidation rate at pH from 9 to 10 and a plateau at a pH range of 10 to 11. However, applying the latter pH range on oxidation of polysaccharides gave unavoidable depolymerization. An example is weight-average degree of

polymerization (DPw) for cellouronic acid prepared by TEMPO/NaClO/NaBr oxidation at pH 10 ranged from 40-80, and were far lower than those of the starting material (the original celluloses DPw 380-1200) (Isogai *et al.* 2009; Shibata *et al.* 2006). The degradation could be attributed to the β-elimination under basic conditions with the assistance of electron-withdrawing groups, such as aldehydes and ketones, and a good leaving group such as an alkoxyl group (de Nooy *et al.* 1996). The presence of carbonyl groups at $C_6$ contributes to increase the acidity of the hydrogen at $C_5$, causing the deprotonation during the alkaline reaction, and thus the formation of a double linkage between $C_4$-$C_5$ and a subsequent cleavage of the group linked to $C_4$ (Fig. 4.6). Hence, the end β-elimination products are comprised of a reducing carbohydrate and an unsaturated carbohydrate (Spier *et al.* 2017). The depolymerization was found to decrease at lower pH values, despite the fact that much longer reaction times are necessary. It may be noted that hypohalite becomes more active and is known as an oxidant for secondary alcohols. Therefore, a lower pH enables the primary oxidant (hypohalite) to act instead of TEMPO (Cosenza e*t al.* 2015). Thus, optimizing the reaction oxidation of polysaccharides would involve a trade-off between the highly selective oxidation of primary alcohols and depolymerization of polysaccharides.

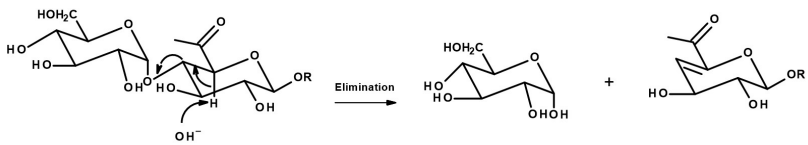


**Fig. 4.6.** Scheme of the mechanism of *β*-elimination

Although this oxidation protocol is synthetically very efficient, it has many environmental and economical drawbacks such as the possible formation of chlorinated by-products in addition to stoichiometric amounts of sodium chloride waste, and the use of bromide as a co-catalyst. Some procedures without the co-catalysis by NaBr have been recently introduced such as an electromediated oxidation (Isogai *et al.* 2010) and an acid neutral process (TEMPO/NaClO/$NaClO_2$) (Hirota *et al.* 2009). Although these systems were reported to yield high carboxylate content, their reaction rates are slow in comparison with that of the bromide assisted TEMPO catalysis. So far, the TEMPO/NaOCl/NaBr system is still widely used for the oxidation of polysaccharides.

### 4.2.3. TEMPO/Laccase/$O_2$

#### 4.2.3.1. Reaction methology

The chemical oxidation of primary alcohols using TEMPO-NaClO-NaBr mediated system produce large amounts of bromide-salt. Environmental concerns have shifted the research interest toward halide-free oxidation systems. Besides alternative routes such as TEMPO/NaClO/$NaClO_2$ system (Hirota *et al.* 2009) and TEMPO electromediated oxidation (Schämann and Schäfer 2003), another promising approach is the use of oxidative enzymes such as laccases, in combination with $O_2$ as primary oxidant. Similar to other TEMPO-mediated systems, the $TEMPO^+$ is generated, but only $O_2$ is consumed and $H_2O$ is the by-product during the reaction (Aracri *et al.* 2011). Additionally, the fact that the reaction operates at near-neutral pH is proposed to reduce the depolymerization caused by the β-elimination reaction, that occurs favorably at pH 10-11.

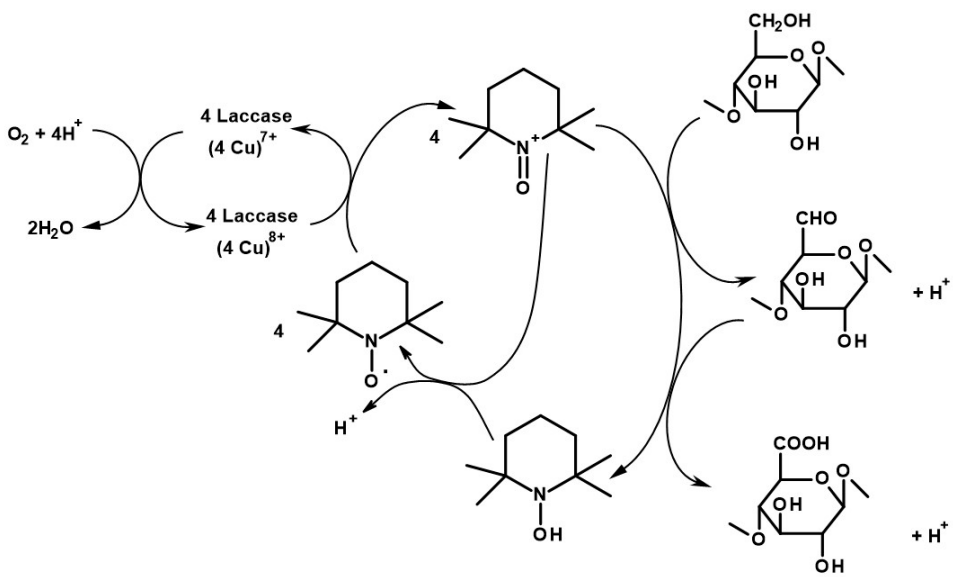


**Fig. 4.7.** Scheme of the mechanism of laccase/TEMPO oxidation of $C_6$-OH groups of cellulose at pH 5

As described in **Chapter 3**. the laccase enzyme family comprises an extended group of blue multi-copper oxidases, known to oxidize a wide range of substrates, mainly phenols and substituted amines, coupled to the reduction of molecular $O_2$ to $H_2O$ (Lavazza *et al*. 2011). The inclusion of TEMPO as a mediator in the oxidation system enables the enzyme to oxidize non-phenolic substrates (e.g. benzylic, aliphatic and propargylic alcohols, carbohydrates). The oxidation reaction proceeds in two steps: the enzyme-catalysed oxidation of TEMPO to form a $TEMPO^+$, which in turn oxidizes chemically and selectively primary alcohol groups to the corresponding aldehydes and acids in a second step. (Banci *et al.* 1999) (Fig. 4.7). While the enzymatic step favors acid pH, because laccase has an optimum between 3.5 and 6.0, depending on the sources (Minussi *et al.* 2007), the chemical step is favored at alkaline pH (Bragd *et al.* 2004). Nevertheless, the pH should be kept in the acidic pH range with respect to laccase activity.

#### 4.2.3.2. Laccase stability

The laccase/TEMPO oxidation is a chemo-enzymatic reaction in which laccase under aeration oxidizes TEMPO to $TEMPO^+$, which subsequently oxidizes alcohols chemically. In the medium with the presence of many chemicals, maintaining enzyme stability is of critical importance, directly affecting the viability of the reaction. Enzymes are made of amino acids and are relatively fragile; their three-dimensional structure is maintained by weak interactions. Therefore, they can be damaged by external influences such as temperature or chemical attack, resulting in loss of their activity. This is called the denaturation of enzymes.

Thus, the presence of $TEMPO^+$ can cause the denaturation of laccase. Under the oxidation condition, $TEMPO^+$ is formed by the laccase-catalysed oxidation of TEMPO. This $TEMPO^+$ is known as strong oxidant, capable of selective oxidation of all kinds of alcohol groups and amino acid residues. In 1998, Isogai *et al* revealed the reaction of primary amine groups of chitosan with TEMPO to form smaller molecules (Isogai *et al.* 1998). Therefore, laccase enzymes containing amino acid residues could be degraded and lose their activity. To confirm the instability of laccase in the presence of $TEMPO^+$, Arends *et al.* (2006a) investigated laccase activity in furfuryl alcohol oxidation under the preincubation of laccase with TEMPO/$O_2$ or TEMPO/$N_2$. The data showed that the laccase activity with incubation under $N_2$ is much higher than that observed with incubation under $O_2$. As a result, a higher accumulation of $TEMPO^+$ in the solution, will cause a considerable reduction in laccase activity. This is evident in a study of Jiang *et al*. (2017), about the oxidation of hardwood bleached kraft pulp. The data showed that with doubled laccase concentration, the carboxylate content of oxidized cellulose

was almost constant. A probable explanation for this is the denaturation of laccase by high $TEMPO^+$ content, formed at high enzyme concentration.

Moreover, any increase or decrease in temperature in enzymatic reactions always results in the significant influence of reaction rate, and denaturation of enzyme. This is more profound in the laccase/TEMPO reaction with the usage of $O_2$ as first oxidant because of the low solubility of $O_2$ at high temperatures. Besides, the accompanied chemical step occurs faster. It is typically assumed that the reaction rate doubles with a temperature increase of 10 °C. Thus, optimizing temperature in the laccase/TEMPO oxidation is of interest. In the study presented in Paper I, three different temperatures were evaluated, concerning the denaturation of laccase from *Trametes versicolor* and the reaction rate. The results indicated that the laccase expressed the highest activity at 40 °C, but was degraded at extended reaction times, leading to a decreased average reaction rate. Good stability was observed at 30 °C. The observation that high temperatures cause a decrease in the yield of the oxidation agrees with the results presented in 2017 by Quintana *et al.*

#### 4.2.3.3. Kind of reactions

***Oxidation of alcohols***

As described above, TEMPO is a selective oxidant, capable of oxidizing primary alcohols in the presence of secondary alcohols. When the laccase/TEMPO system is applied on carbohydrates, hydroxyl groups at $C_6$ are converted into corresponding carbonyl and carboxylic groups. Thus, the efficiency of the oxidation can be evaluated by monitoring the formation of aldehydes and acids. In accordance with some studies on the oxidation of carbohydrates, during the initial phase, a steep increase in aldehyde content was observed without concomitant acid formation, which increased mainly in the later phase, when aldehyde formation levelled off (Patel *et al.* 2011; Paper II). This indicates that the oxidation process of alcohol to aldehyde proceeds much faster than aldehyde to acid. Interestingly, the oxidation behavior is in contrast with a normal routine in almost of chemical oxidation and is only evident in the application on carbohydrate with high degree of polymerization, not on glucose (Paper I). The question of whether there is any hindrance to TEMPO attack toward aldehyde during the oxidation process has been studied. Potthast *et al.* (2007) found that the aldehyde groups at $C_6$ form intra- and intermolecular hemiacetal linkages with hydroxyl groups of carbohydrate and/or aldehyde hydrates. This can be interpreted as steric hindrance of TEMPO during oxidation of aldehyde to acid, and thus a decrease of the further oxidation to carboxylic groups.

Besides, despite no report on the laccase/TEMPO oxidation of secondary alcohols in carbohydrates, the oxidation on small molecular compounds displayed some side-reactions, involving secondary alcohols which are oxidized to ketones (Fabbrini *et al.* 2001; Gross *et al.* 2014, Paper II). An explanation could be that under acidic condition, adducts formed between alcohol and $TEMPO^+$ was via a linear transition state (section 4.2.1), leading to a reduction in steric hindrance and less selectivity between primary and secondary alcohols. However, it is worth noting that the TEMPO still shows predominant transformation of primary alcohols over secondary ones. Arends *et al*. (2006b) investigated the reactivities of TEMPO for benzyl alcohol and 1-phenylethanol, and showed that the conversion of benzyl alcohol was 3 times faster than that of 1-phenylethanol. Besides, the use of high TEMPO concentration will enhance the formation of both aldehyde and ketone (Paper II). Liebminger *et al.* (2009) optimized the laccase-catalysed oxidation of glycerol. In this case, low TEMPO concentrations led to low conversion and the predominant product from the primary alcohol oxidation (glyceraldehyde). At high TEMPO concentrations, the oxidation was driven faster and accompanied by high generation of ketones followed by the complete transformation to aldehydes.

***Depolymerization***

As compared with the chemical approach (TEMPO/NaClO/NaBr), the laccase/TEMPO approach also shows a similar degree of molecular weight loss, a major drawback of the TEMPO-mediated system (Patel et al. 2010; Quintana et al. 2017; Paper II and III). It is known that in the chemical oxidation, the degradation is mainly due to alkali-induced β-elimination processes starting from carbonyl groups (Potthast *et al*. 2009). However, in the case of laccase-catalysed oxidation, near-neutral pH medium is used and should hinder β-elimination. Therefore, Patel *et al*. (2010) suggested that the degradation probably takes place through a homolytic process, with some radical species formed as by-product during the oxidation. Recently, Aracri *et al.* (2012) revealed a linear relationship between the aldehyde content and depolymerization level and confirmed that the loss of molecular weight is correlated to the presence of aldehyde in the carbohydrate. The observation is in agreement with the results obtained in Paper II.

# 5. Laccase/TEMPO oxidation of alkyl glycosides

AGs are sugar-based nonionic surfactants possessing attractive properties, especially AGs with a long hydrophobic chain. However, their wide application is limited because of their low solubility. Thus to increase solubility, many chemical and enzymatic synthetic methods have been conducted to convert primary alcohols of glycosides to acids (**Chapter 4**). Environmental concerns have shifted the research interest towards the laccase/TEMPO system, in which one of the highlights is the use of the oxidative enzyme as laccase in combination with $O_2$ as primary oxidant. In this chapter, the TEMPO-mediated oxidation of primary alcohols of AGs in the presence of laccase is presented in detail with OG, DDM, HDM, and DDMO as substrates. All parameters such as the concentration of laccase, TEMPO and substrates, the structure of substrate, and the efficiency of aeration of the reaction solution are of great importance. They need to be optimized to obtain sufficient conversion.

## 5.1. Influencing factors on the oxidation

### 5.1.1. Oxygen supply

During the oxidation of alcohol to acid, $O_2$ is consumed. A supply of $O_2$ is thus required. Moreover, the solubility of $O_2$ is low at high temperature. Therefore, most all laccase/TEMPO oxidation are performed at room temperature to avoid the limitation of $O_2$. Paper I describes the laccase/TEMPO oxidation of OG with two ways to supply $O_2$ into the reaction solution, comprising of bubbling with pure $O_2$ and shaking the mixture in normal atmosphere. It was concluded that running the reaction under a normal atmosphere yielded a similar conversion of alcohol into aldehyde as pure $O_2$, which means that laccase was generally saturated with $O_2$ under both reaction conditions. This is in line with a previous study of the oxidation of benzyl alcohols (Arends *et al.* 2006b). Moreover, $O_2$ is supposed to enhance the

oxidation of aldehyde to acid together with TEMPO (Jausovec *et al.* 2015; Li *et al.* 2013). As discussed in **Chapter 4**, TEMPO oxidation proceeds in two steps from alcohol to aldehyde and aldehyde to acid. With the sufficient supply of $O_2$ for the reaction, the latter step seems to occur fast. This was also reported in the oxidation of starch with the double carboxylic content compared with carbonyl content when bubbling with $O_2$ (Mathew and Adlercreutz 2009).

### 5.1.2. Laccase type and concentration

To date, the TEMPO-mediated oxidation of carbohydrates has been described widely using a variety of laccases. As discussed in **Chapter 3**, laccase belongs to a family of multi-copper oxidases that are widespread in numerous fungi. They can accept a wide range of substrates, such as alcohol, phenol, aromatic amines and thiol compound, when in association with a mediator such as TEMPO. The ability of laccase to oxidize TEMPO depends on the redox potential of the enzyme, which was reported not to exceed 800 mV. Scanning four commercially available laccases on TEMPO oxidation, the enzyme from *Myceliophthora thermophilia* has low or no oxidation activity because of its low redox potential of 450 mM. Meanwhile, the laccase from *Trametes versicolor* (laccase TvL) with a high redox potential of 785 mM has a much higher activity than those from *Thielavia Arenaria* and *Cerrena unicolor*. In the case of the two latter, commercial preparations displayed the presence of a measurable amount of mannanase side activity, causing unwanted enzymatic hydrolysis of the polymers. On the other hand, laccase TvL did not exhibit mannanase side activity, and is thus the enzyme of choice of oxidation of AGs, (Lavazza *et al.* 2011; Mathew and Adlercreutz 2009).

In the oxidation reactions, at small scale performed at ambient atmosphere, the $O_2$ content in the solution was low, limiting the laccase concentration used. In Paper I, the laccase concentration was investigated by the comparison of initial reaction rates, which are determined by the conversion of OG. Theoretically, the initial reaction rate in an enzymatic reaction is expected to be proportional to the enzyme concentration. However, this was not completely applicable in the oxidation of OG. The initial rate was only proportional to enzyme concentration at low enzyme content, and plateaus at high enzyme content. This suggests the influence of $O_2$ limitation in the reaction. Another explanation is that the use of the high laccase concentrations enhances the formation of oxidized TEMPO, which is known to inactivate laccases to some extent (**Chapter 4**) (Mathew and Adlercreutz 2009; Jiang *et al.* 2017).

### 5.1.3. TEMPO concentration

In addition to the type and concentration of laccase, TEMPO plays a vital role to drive the oxidation of alcohol. $TEMPO^+$ formed by the oxidation of TEMPO with laccase not only drives conversion of alcohol to corresponding aldehyde, and acid but also inactivates laccase. Hence, an investigation of the TEMPO influence is of profound importance.

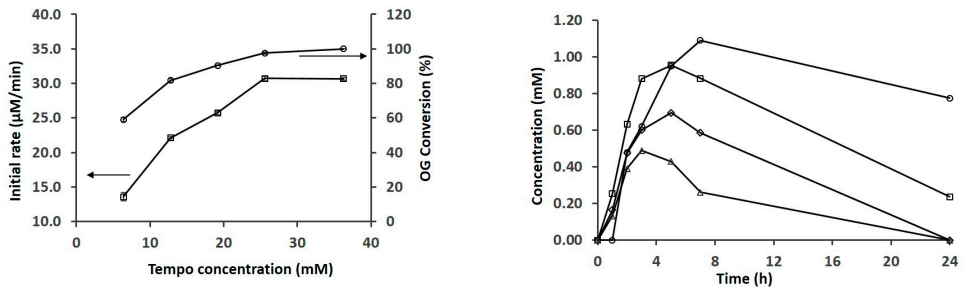


**Fig. 5.1.** The influence of TEMPO on the initial rate, conversion of OG, and the formation of aldehyde after 24 hours' reaction time (Paper I). Reaction conditions: OG (20 mM), laccase (0.333 mg/ml) and different TEMPO concentrations (● 6.2 mM, ■ 12.8 mM, ♦ 25.6 mM, ▲36.3 mM) at 24 °C with shaking at 750 rpm (Paper I).

Paper I presented that the influence of TEMPO at low to moderate concentrations on the oxidation rate of OG appeared to obey Michaelis Menten kinetics with a $K_{M,app}$ value of 12.8 mM and $V_{max}$ of 43.6 µM/min. However, at higher TEMPO concentrations, the initial rate was constant at 30.9 µM/min (Fig. 5.1). Quintana *et al* (2017) also reported a similar result in the oxidation of pulp with increased TEMPO concentrations. The result showed that oxidation with TEMPO doses of 2% and 8% caused a laccase inhibition of 31 and 80%, respectively. However, in the case of OG oxidation, full conversion of OG was still obtained at high TEMPO concentration, thus indicating that the extent of enzyme inactivation was moderate under the conditions used.

Moreover, the concentration of TEMPO has an influence on the number and type of products. In the case of OG oxidation (Paper I), TEMPO appears to be an efficiently selective oxidase to generate two sole products comprised of OG-CHO and OG-COOH. In the study presented in Paper I (Fig. 5.1) a strong correlation can be seen between the increase in TEMPO concentration and the decrease in OG-CHO content, indicating that TEMPO promotes the aldehyde oxidation (Marjasvaara *et al.* 2004) and a high TEMPO concentration favors the full oxidation to acid. A yield

of 100% of OG-COOH makes the laccase/TEMPO system highly competitive among the OG oxidation methods (Boelrijk *et al.* 1996; Ferlin *et al.* 2008).

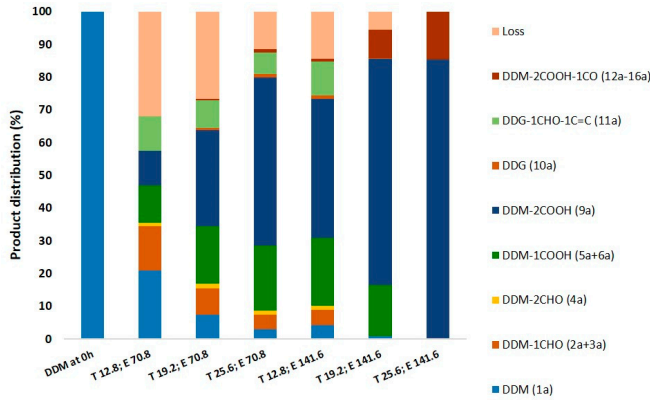


**Fig. 5.2**. Product distribution of DDM oxidation at 24 hours with different TEMPO and laccase concentrations. Reaction conditions: DDM (20 mM), different concentration of laccase (E, 70.8 U/µl and 141.6 U/µl) and TEMPO (T, 12.8 mM, 19.2 mM, 25.6 mM) at 24 °C with shaking at 750 rpm (Paper II).

In the case of DDM oxidation (Paper II), three types of reactions were detected, including the oxidation of primary and secondary alcohols, and degradation of the carbohydrate chain. These are the typical reactions in the oxidation of polysaccharides, which were thoroughly discussed in **Chapter 4**. In Paper II, a significant influence of TEMPO concentration on these three reactions was observed (Fig. 5.2). Increasing TEMPO concentration leads to the substantial generation of the expected product DDM-2COOH, accompanied by a slightly increased conversion of secondary alcohols and a considerable decrease in degradation. The latter is attributed to the β-elimination-like reaction in association with the presence of aldehyde groups in the molecule. The degradation rate is assumed to be proportional to the aldehyde content in the polysaccharide (**Chapter 4**). The aldehyde can be converted rapidly to acid at high TEMPO concentration. Moreover, although TEMPO is demonstrated to be less selective in acidic medium, the ratio of primary to secondary alcohol oxidation rates is rather high, and the formation of ketone groups only occurs at high TEMPO concentrations. Thus, to achieve the highest yield of the diacid product with a low extent of side-reactions, it is necessary to conduct the oxidation reaction at high TEMPO and enzyme concentrations with optimized reaction time. In the study presented in Paper II, a yield of 85% of the diacid at 25.6 mM TEMPO and 141.6 U/µl laccase was obtained at 24 h, which is a quite competitive result as compared with chemical oxidation

processes with metal catalysts (Vinke *et al.* 1992; Boelrijk *et al.* 1996), and even the TEMPO-mediated anodic oxidation of methyl disaccharide (Schämann and Schäfer 2003 and 2005).

### 5.1.4. The influence of alkyl glycosides

AGs are non-ionic surfactants with alkyl tail as hydrophobic part and carbohydrate head group as hydrophilic part. The behavior of the surfactants in aqueous solution depends to a large extent on concentration of AGs and the length of the alkyl chain, while the extension of carbohydrate chain improves their solubility. Thus, it is important to evaluate the effect of these parameters on the laccase/TEMPO oxidation reaction.

***Carbohydrate chain***

Generally, the extension of the carbohydrate chain affects the kind of reactions and the nature of products. The process exhibits a significant selectivity for the primary alcohol in alkyl glucoside oxidation, in which there is the formation of aldehyde and acid (Paper I). Once the number of glucose units in the hydrophilic chain increases, the behavior of TEMPO changes, along with increased degradation (**Chapter 4**). In the study presented in Paper II, the formation of intra- and inter-hemiacetal linkages between aldehyde groups at $C_6$ and hydroxyl groups in sugar rings might limit the accessibility of TEMPO to convert carbonyl groups to carboxylic group (Potthast *et al.* 2007; Rohrling *et al.* 2002). This behavior of the TEMPO/laccase system is completely the opposite compared to what was found in the oxidation of OG. Interestingly, the reactivity of TEMPO is proportional to the length of the carbohydrate chain. Paper III describes the predominant conversion of DDMO over DDM in the same solution. This might be due to a steric hindrance by the alkyl chain on TEMPO attack. The fact that with more glucose units being further away from the alkyl chain enables the conversion to proceed more rapidly.

Moreover, the degradation favors the cleavage of glucose-1,4-glucose bonds that are associated with carbonyl groups; thus, the loss of molecular weight is inevitable and severe when applying this oxidation system on AGs with long carbohydrate moiety. In the DDM oxidation, the maximum degradation detected was about 40% (Paper II). In the oxidation of DDMO, besides oxidation products, degradation products with the elimination of 1 to 6 glucose units were also obtained to a relatively high extent (Fig. 5.3). Quintana *et al.* (2017) reported about 85% of degree of polymerization loss in the TEMPO-mediated oxidation of pulp samples with the presence of laccase. Also, the conjugation of the aldehyde group and the C=C double bond in degradation products excludes the hydration of aldehyde in water.

Thus, the conjugated aldehyde is not oxidized further to acid (Paper III) (Quintana et al. 2017).

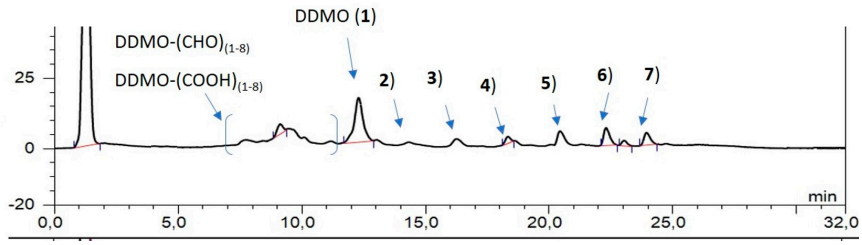


**Fig. 5.3.** HPLC-CAD chromatogram of the oxidation of DDMO (15 mM) using the laccase (141.7 U/L)/TEMPO (25.6 mM) system at 5h (**1A**), 11h (**1B**), and 24h (**1C**). Identification of peaks was done by HPLC-MS.. Peaks of 2 to 7 represent degradated products (Paper III)

### *Alkyl chain*

Depending on the length of the alkyl chain and concentration, AGs can appear in the form of monomers, micelles or lamellar structures in water. A previous study reported the effect of micellar structure on the selective oxidation of two primary hydroxyl groups of decyl maltoside. No oxidation toward dicarboxylic acid was observed because the $C_6$ in the inner glucose unit cannot be reached by the Pt catalysts employed (Vinke *et al*. 1992). However, in the laccase/TEMPO oxidation system, whatever form the AGs existed in, the reaction worked well to achieve high yields of uronic acids. Typically, the oxidation of 60 mM OG (CMC 22 mM) gives high conversions of 85% after 24h, while 20 mM DDM (CMC 0.2 mM) is converted completely to furnish a yield of 85% of diacid (Paper I and II).

In addition, increasing the length of the alkyl chain in AGs will lower their solubility, even make them insoluble. For example, HDM having a very low CMC value of 2.3 µM (Larsson *et al.* 2019) is almost insoluble in water at room temperature, resulting in a gel-like heterogeneous reaction solution. Thus, it will be challenging to conduct the oxidation of these AGs in homogeneous solution. Paper II describes the oxidation process of 40 mM HDM with and without co-solvent, which was used to improve the solubility of HDM. Surprisingly, after 24 hours, the reaction without co-solvent gave a HDM conversion of about 99%, compared with 84-87 % obtained in the reactions with co-solvent. The latter results could be attributed to the effect of viscosity limiting mass transfer. The attempt to dissolve HDM in water by using co-solvent caused an increase in the solution viscosity, thus lowering enzyme activity. It is worth noting that the transformation of carboxylic groups from primary alcohol groups increase the water solubility: oxidized HDM has much higher solubility than the substrate HDM.

## 5.2. Physical properties of OG-COOH

### 5.2.1. Micelles and micellization

As described in **Chapter 2**, micelles are formed when the surfactant concentration is above the CMC and surface tension keeps constant, bringing sudden changes to all properties of the solution. Therefore, many researches focus on micelle formation, to be able to take advantage of this phenomenon. The micelle formation is proposed to be governed by the competition between two forces: the hydrophobic interaction between tails, which provides the driving force for aggregation, and the electrostatic or steric repulsion between the head groups, which limits the size of the micelles (Joshi *et al.* 2002). Thus, it is expected that the formation and the size of micelles are dependent on the structure of the surfactant and the charge on the head group.

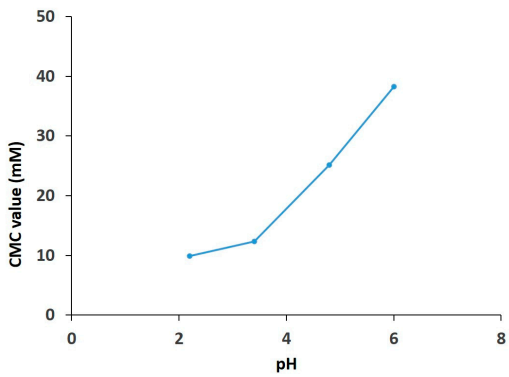


**Fig. 5.4.** CMC of OG-COOH at various pH values. The measurement was done at ambient temperature and the pH was maintained by citrate-phosphate buffer (Paper IV).

Carboxylated derivatives of AGs are weakly ionizable, thus they will exhibit a pH dependent behavior. Let us consider the behavior of OG-COOH in solution based on the investigation of CMC and micellar size (Paper IV). The introduction of carboxylic groups can turn the surfactant into either nonionic or anionic form, modulated by pH, probably changing the behaviour and properties of the surfactant solution. Unlike normal anionic surfactants such as fatty acids, the OG-COOH does not precipitate at pH below their pKa values (Kanicky *et al.* 2000). On the other hand, the deprotonation of these carboxylic groups on AGs at pH values above pKa can cause the predominance of the electrostatic repulsion between head groups over the hydrophobic interaction, leading to limited micellization and increased CMC

value. In particular, when pH of the OG-COOH solution increased from 2.2 to 6.0, the content of charged OG-COOH increased, resulting in strong repulsive forces and a dramatic increase in CMC (Fig. 5.4) (Paper IV).

In addition to CMC, micelle size is of considerable interest. It is known that the growth of micelles is dependent on the salt effect. To ionic surfactants, counter ions of salt tend to stay close to charged head groups, decreasing repulsive interactions between head groups. Meanwhile, for nonionic surfactants, the outer mantle of the micelle is protected through hydration. The added salt in solution can cause a so called salting out effect, meaning that the hydrophilic head groups are dehydrated and thus assist self-aggregation of the surfactant and increase the micellar size (Rosen and Kunjappu 2012; Ren *et al.* 2010; Umlong and Ismail 2007; Zhang *et al.* 1996). However, both these salt effects were observed in the investigation of micellar size formed by OG-COOH at pH of 2.2 and 4.7. Indeed, at both the two used pH values, the OG-COOH solution is a mixture of charged and uncharged surfactants, in which at pH 4.7, charged forms of OG-COOH dominate, thus, the increase in micellar size by added salt might be attributed mainly to the repulsive effect. On the other hand, with mainly uncharged OG-COOH in the solution at pH 2.2, the growth of micelles was probably due to the salting out effect. Consequently, there is an evident increase in the size of micelles formed by OG-COOH (Paper IV) with increasing salt concentration.

### 5.2.2. Foamability

Foamability is a property inherent to surfactants. Applications for foam occur widely in many fields such as food industry, mining, fire-fighting products and pharmaceuticals (Kontogeorgis and Kill 2016). However, foaming quite often cause undesired side effect in various processes. For instance, in the paper-making process, it is necessary to use a surfactant which has surface activity, but does not produce much foam (Rosen 2004). Therefore, the foaming of surfactants has attracted considerable interest in both the industrial and academic fields. Paper I described the evaluation of foamability of OG-COOH through two factors – foam height and the decay of foam with time. Having a carboxylic group in the molecule, OG-COOH with pKa of 3.32 is expected to exhibit a pH dependence on its foaming. The data showed that there was a reduction in foamability of OG-COOH as increasing pH from 2.2 to 5 and no foam was observed at pH above 5 (Fig. 5.5). Interestingly, this trend opposes that from OG that forms stable foam from pH 6 to 10 (Bergeron *et al.* 1996). It was concluded that the different foaming of these two surfactants is due to the introduction of carboxylic group. The fact that the deprotonation of carboxylic group of OG-COOH increased with increasing pH gave rise to electrostatic repulsion between charged head groups. This led to a weak and transient foam (Kanicky *et al.* 2000). Furthermore, the behavior of OG-COOH

toward foaming is different from that of classic carboxylated surfactants, for example fatty acid salts such as sodium laurate, which show a maximum foam height when pH is around its pKa (Kanicky *et al*. 2000). This is explained to be due to the ion-dipole interaction between the carboxylic and carboxylate forms of fatty acid at pH around pKa. However, in the case of OG-COOH, this interaction might be hampered by the presence of the pyranose ring. In conclusion, the foaming of OG-COOH is markedly dependent on pH, and its low foamability can be interesting in applications where no foam is required.

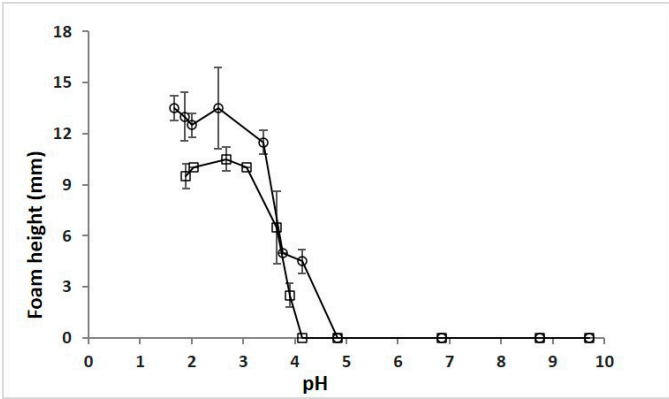


**Fig. 5.5.** Plot of the initial foam height as function of pH value for OG-COOH concentration of 10 mM (■) and 20 mM (●). The pH was adjucted by adding 2 M HCl or 2 M or 6 M NaOH solution (Paper I).

# 6. Enzymatic elongation of anionic alkyl glycosides

AGs are biodegradable surfactants and find many applications in dishwashing, laundry detergents, cosmetic and cleaning products. However, so far, many modifications to their structures have been studied to increase their application in other fields such as pharmaceuticals, where more benign compounds are strictly required. One route of particular interest is the elongation of the carbohydrate part of AGs. The extension of AGs was reported by using different enzymes (Svensson *et al*. 2009a and b). Of the enzymes employed, cyclodextrin glycosyltransferases (CGTase) appears as a potential candidate and is capable of glycosylating a wide variety of substrates. They are known as multi-function enzymes, which catalyse four types of reactions: cyclization, coupling, disproportionation and hydrolysis (Van der Veen *et al.* 2000), in which their hydrolyzing activity is weak. This chapter reports the CGTase-catalysed synthesis of anionic AGs with long oligomeric head group and the key factors influencing the yield of the transglycosylation reactions.

## 6.1. Characterization of CGTases

CGTases belong to an α-amylase family (glycoside hydrolases family GH13), which act on starch and α-glucan (Henrissat 1991). CGTases are composited of five domains (A-E) in which domains A-C are present in all enzymes in the GH13 (Lawson et al. 1994; Klein and Schulz 1991). A is the catalytic domain and contains the proton donor Glu 257, and the bases Asp 229 and Asp 328 (numbering by CGTase from Bacillus circulans strain 251). Together with domain A, domain B forms a groove on the enzyme surface, where the binding of substrate takes place through hydrogen bonding with hydroxyl groups of the carbohydrates at several subsites. Being positioned after the domain A and on the opposite side of B, domain C is suggested to assist in raw starch binding. The additional domain E has the same function as C, while the function of domain D is unknown. According to analysis of the structure of the CGTase enzyme from *B. circulans* strain 251, substrate binding in the groove occurs in subsites with the numbering from + 2 to -7, where subsites labeled "+" bind the acceptor and the other ones bind the donor (Davies et al. 1997).

The catalytic site is between subsites + 1 and -1 and consists of conserved amino acid residues.

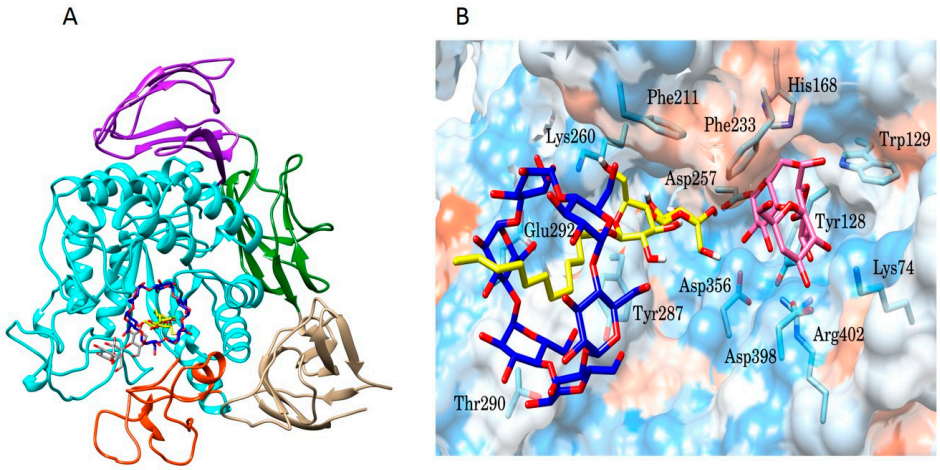


**Fig. 6.1.** Molecular model of the complex Toruzyme (CGTase from *Thermoanaerobacter* sp. ATCC 53627)/DDM/α-CD. Panel A represents the homology model of Toruzyme. Domain A is in cyan, domain B in orange, domain C in purple, domain D in green, and domain E in tan. Panel B represents the predicted interactions between Toruzyme and ligands. The α-cyclodextrin is represented with blue sticks, carbon atoms in blue and oxygen in red. Dodecyl maltoside carbons are in yellow with oxygens in red. Maltotrioside covalently bonded to aspartate 257 is represented with carbons in gray with oxygens in red. Hydrophilic surfaces are represented in blue and hydrophobic in orange. (Paper III).

The binding of acceptor and donor on the enzyme was illustrated based on the molecular model of the complex *Thermoanaerobacter* sp. ATCC 53627 CGTase (Toruzyme) and DDM as acceptor and α-CD as donor (Fig. 6.1). The donor is bound to glycon subsites by hydrogen bonds with amino acids involving His 168, Asp 356, Trp 129, Asp 398, Arg 402, Lys 74, Asp 398. Meanwhile, the acceptor gives a smaller number of hydrogen bond interactions with aglycon subsites with the assistance of amino acids – Phe 223, Phe 211, Tyr 287, Lys 260. These interactions are more hydrophobic than that in glycone subsites (Paper III).

Furthermore, the acceptor specificity of CGTase enzyme is also demonstrated by interactions of several regions of the active site groove. A complex of γ-cyclodextrin with a crystal structure of a CGTase from *B. circulans* strain 251 revealed different hydrophobic interactions of linear and cyclic compounds with Phe 183 and Phe 259. Phe 259 plays a role for stacking interactions with cyclic compounds, whereas for Phe 183, the interactions are better with linear substrates (van der Veen et al. 2001).

## 6.2. Reactions catalysed by CGTases

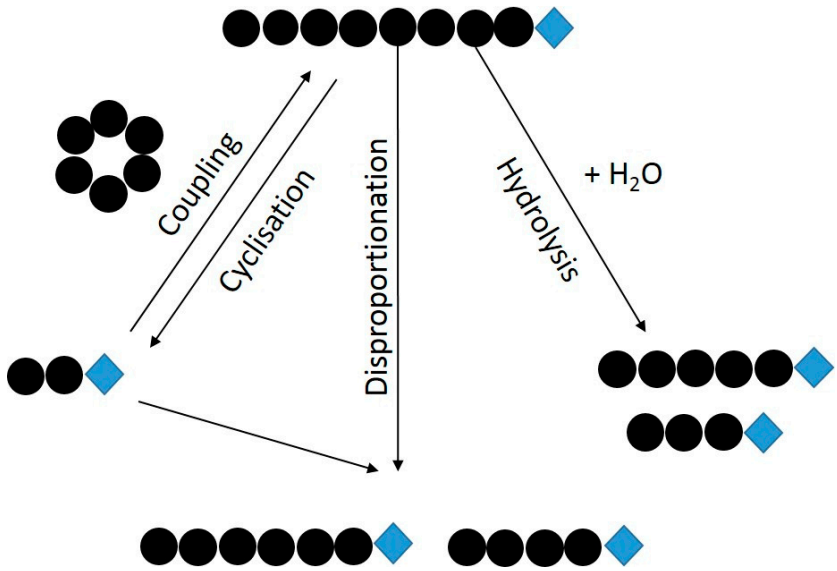


**Fig 6.2.** Schematic illustration of the CGTase-catalysed reactions. The dark circle represents glucose unit, the blue iozenge represents the other oside as acceptor.

CGTases catalyse four different reactions including cyclization, coupling, disproportionation and hydrolysis (Fig. 6.2). The process consists of two steps; in the first step, the action of a CGTase starts with the cleavage of an α-1-4-linkage of the substrate between the -1 and + 1 subsite to yield the covalent intermediate. In the second step, the newly produced intermediate is transferred to an acceptor, which can either be a nonreducing end of another carbohydrate molecule (disproportionation) or its own nonreducing end (cyclization) or water (hydrolysis). CGTases also catalyse the reverse reaction of cyclization, in which α-CD is opened and transferred to a linear acceptor (coupling). Based on cyclization reaction, these enzymes produce three types of cyclodextrins with different degree of polymerization of 6, 7, and 8. The disproportionation and coupling reaction have been applied in glycosylation of stilbenoids (Mathew *et al.* 2012), AGs (Svensson *et al.* 2009a and 2009b) and 2-O-α-D-glucopyranosyl-L-ascorbic acid (Gudiminchi *et al.* 2016). However, the predominance of disproportionation or coupling is highly dependent on the kind of enzyme and their substrate specificity (Paper III) (Rather *et al.* 2015).

## 6.3. Synthesis of anionic AGs with oligomeric head group

Due to the unique ability of CGTases to catalyse a variety of reactions, they are the enzymes of choice of the synthesis. Specially, two acceptors (OG-COOH and DDM-2COOH) employed in Paper III consist of carboxylic groups, which are deprotonated at pH above 3 and able to affect the enzyme activity. Thus, the influence of different parameters will be discussed in detail below with emphasis on kinds of donors and acceptors and reaction conditions.

### 6.3.1. Donor type and concentration

In previous studies, the donor employed mainly in the elongation reaction of AGs was α-CD. As described above, CGTase can catalyse several different reactions simultaneously depending on the kind of substrate present in the reaction mixture. As the formation of the first coupling product increases, there can be a competition between the coupling product and α-CD, in binding at the donor site of the enzyme (Paper III). This facilitates the initiation of disproportionation and cyclization reactions, resulting in a diversity of products. The effect becomes obvious when the initial donor concentration decreases (Svensson and Adlercreutz 2011).

Moreover, α-CD is known to be able to form inclusion complexes with hydrophobic molecules, in which the molecules can be trapped. Some studies take advantage of the complex of α-CD in the glycosylation of hydrophobic molecules with CGTases in water medium (Marié *et al.* 2018). However, Zehentgruber *et al.* (2011) proved that the CGTase enzymes seem not to favor the α-CD/DDM inclusion complex as donor, and only free α-CD is important for the reaction. As shown in the two mentioned cases, it is beneficial to utilize high α-CD concentrations, avoiding the production of diverse products, and providing free α-CD for the enzyme. In the same study, Zehentgruber *et al.* (2011) recommended the use of a ratio of α-CD and DDM concentrations above 4, which was applied to the synthesis in Paper III.

### 6.3.2. Acceptor type

The acceptor specificity of CGTases has been determined by evaluating the binding of an acceptor to the acceptor site of the enzyme (Van der Veen *et al.* 2001). The binding is highly dependent on the structure of the acceptor. Some studies on the coupling reaction have revealed that maltose has an optimal length (Wongsangwattana *et al.* 2010). It could be due to the two acceptor binding subsites usually present in CGTases. This is in agreement with the result in Paper III where the conversion of OG-COOH is somewhat lower than that of DDM-2COOH as

utilizing either CGTase from *Bacillus macerans* (Amano) or *Thermoanaerobacter* sp. (Toruzyme) as catalysts, and α-CD as donor.

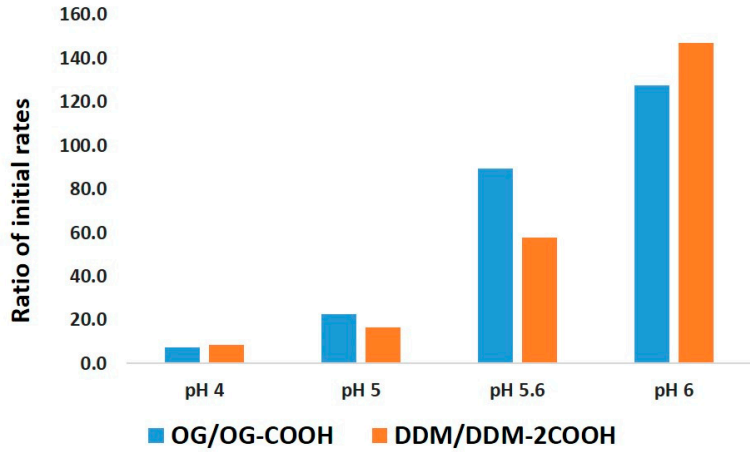


**Fig. 6.3.** pH dependence of the CGTase catalysed elongation of OG, OG-COOH, DDM and DDM-2COOH as the ratio of initial rates. Reaction parameters: OG (15 mM)/ OG-COOH (15 mM)/ DDM (15 mM)/ DDM-2COOH (15 mM), α-CD (120 mM), enzyme amounts within the range 1-20 µl, pH 4-6, 60 °C, shaking with 750 rpm (Paper III).

Paper III compares the acceptor efficiency of four different substrates: OG, DDM, OG-COOH, and DDM-2COOH with Toruzyme as catalyst. The two latter are supposed to be deprotonated at the reaction condition (pH 5.6). The fact that the conversion of noncarboxylated substrates was 60 – 100 times faster than that of carboxylated ones indicated that the enzyme favors the neutral form over the negatively-charged form of carbohydrates. This behavior was explained based on analyzing the molecular model of Toruzyme in complex with DDM/DDM-2COOH as acceptor and α-CD as donor. As mentioned above, acceptor subsites show hydrophobic interactions with DDM. In a similar way, DDM-2COOH could be bound at subsite +1 through a hydrogen bond with Lys 260. However, in the reaction condition (pH 5.6), the carboxylic groups of DDM-2COOH were deprotonated to give the ionized form, which can cause electrostatic repulsion with the negatively charged catalytic base, Asp 356. This interrupts the reaction. Thus, a more acidic medium where acceptors DDM-COOH and OG-COOH exist as neutral forms will probably be more favorable. On the other hand, the optimal pH for Toruzyme is known to be at pH 6 (Zhou et al. 2010). Therefore, the elongation of OG-COOH and DDM-COOH is expected to be strongly pH dependent. Indeed, the ratios of the conversion of DDM/DDM-2COOH and OG/OG-COOH decreased with decreasing

pH in the entire range, with 120-150 times at pH 6 and about 8 times at pH 4 (Fig. 6.3).

Many previous studies showed that CGTase enzymes can catalyse the elongation with acceptors having long sugar chains. However, the efficient performance of the acceptors decreased with increasing sugar-chain length, which can in turn be due to the competition for donor subsites with α-CD (Vetter and Thorn 1992; Yoon and Robyt 2006; Bender 1982). In the elongation of DDM-2COOH with α-CD catalysed by Toruzyme, the primary coupling product (DDM-2COOH-6G) is a potential donor in the disproportionation reaction with DDM-2COOH to provide a complex product mixture with AGs having at least between 1-6 extra glucoses (Paper III). As an acceptor, AGs were revealed to be highly efficient if they contain two glucose units (Svensson *et al.* 2009b). However, in Paper III, the concentration of the donor substrate obtained passed through a minimum and increased again towards the end of the reaction. This indicates that DDM-2COOH is a poor acceptor in the competition with other potential acceptors (DDM-2COOH-G2, DDM-2COOH-G6, etc.).

# Concluding remarks

In this doctoral work, several aspects involving the synthesis of anionic AGs with oligomeric head groups and characterization of modified products were addressed. To produce anionic AGs, the selective oxidation in the presence of laccase as catalyst and TEMPO as mediator was conducted. The method is attractive from environmental point of view, because only $O_2$ is used as primary oxidant, producing the by-product $H_2O$. The experimental data showed significant effects of TEMPO and laccase concentration on all aspects of the reaction. Using increased concentrations of both these factors made the reaction faster, and high conversion of primary alcohol to corresponding acid possible and minimized the degradation of carbohydrate, but simultaneously initiated secondary alcohol oxidation. However, the rather high ratio of the oxidation rates of primary and secondary alcohol in association with optimizing the reaction time rendered the formation of by-product ketones limited. The degradation of the carbohydrate chain is the main and inevitable disadvantage of the laccase/TEMPO method, which becomes severe when applying this method on AGs with long carbohydrate chains.

The introduction of carboxylic groups in AG molecules results in significant changes in the physical properties of the surfactant. To AGs with short alkyl chain, the presence of carboxylic groups can give a negative effect on their surface properties, due to increased repulsion between ionized head groups at high pH. This caused an increase in CMC values and a decrease in foamability with increasing pH values. On the other hand, it is evident that the solubility of oxidized AGs is much higher than that of the original AGs, especially long alkyl chain AGs, which have attractive properties, but are limited in use due to their low solubility. This can open up new applications for AGs.

The elongation of anionic AGs with CGTase as catalyst and α-CD as donor was conducted with the aim of generating anionic AGs, having lower cellular toxicity. It was concluded that the enzyme preferably accepts the neutral form of the AG, rather than the ionized one. The strong pH dependence of substrate ionization and enzyme activity must be taken into consideration. Although slower, the reaction proceeded successfully, producing anionic AGs with a varied degree of polymerization in the head groups. Keep in mind that most commercially available AGs are complex mixtures that are known to have useful properties. Therefore, the

production of a mix of coupling products can provide a promising candidate for the surfactant market.

The results of this thesis work provide processes for the synthesis of anionic derivatives of AGs. The methods employed were applied to these types of substrates for the first time and detailed data about the influencing factors on the reactions were obtained. The first observations of the properties of these products could pave the way to a promising novel generation of AGs.

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