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Studies on Hexuronosyl Xylooligosaccharide Degrading Enzymes from Paenibacillus species

A Dissertation Submitted to
the Graduate School of Life and Environmental Sciences,
the University of Tsukuba
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Bioresource Engineering
(Doctoral Program in Appropriate Technology and Sciences for Sustainable Development)

Krisna SEPTININGRUM
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Chapter 1

General Introduction

1.1 Background

Chemical pulping is used for most of paper producing commercially in the world. Chemical pulps are made by cooking the raw material using kraft (sulfate) and sulfite process. The kraft process is the dominant pulping process. In this process, wood chips are digested at elevated temperature and pressures in "white liquor" that contain sodium sulfide and sodium hydroxide as active cooking chemicals. The white liquor chemically degrades and dissolves the lignin that binds the cellulose fibers together and makes the lignin more soluble. This cooking process is applicable to all types of wood. Kraft pulp possesses superior pulp properties in comparison with sulfite pulp. Kraft process produces a variety of pulp used mainly for packaging and high strength papers and board.

Delignification process in kraft cooking is accompanied by the simultaneous dissolution of carbohydrates that has a significant effect on the amount and structure of wood carbohydrates especially hemicelluloses. Structure of hemicelluloses is modified as a result of partial degradation of side-groups in the high alkalinity and temperature of the cooking liquor [1, 2]. More than 50% of the wood hemicelluloses can be dissolved in the kraft liquor. Removal of carbohydrates from the wood solid matrix is due to dissolution of low molecular weight chains, end-initiated depolymerization (primary peeling) and alkaline hydrolysis of glycosidic bonds, which leads to secondary peeling and decrease in the polymerization degree [1].

In this alkaline media, structure of acidic hemicelluloses also modified. Uronic
acid (4-\(O\)-methylglucuronic acid, MeGlcA) side group on the xylan is partly removed from the backbone. Then remaining MeGlcA is converted to 4-deoxy-\(\beta\)-\(\text{L-}\,\text{threo}\)-hex-4-enopyranosyluronic acid (hexenuronic acid, HexA) by the \(\beta\)-elimination of the 4-\(O\)-methoxyl group, after the loss of the hydrogen atom attached to the fifth position of the glucuronic acid residue [2].

Occurrences of HexA groups are significant because of their role in the bleaching process and their influence on the final pulp properties (brightness). HexA contributes to the measured kappa number [3, 4], increases the consumption of electrophilic bleaching agents such as chlorine dioxide, ozone peroxide and peracetic acid in subsequent bleaching agents [5], increases brightness reversion [6], retains metal ions [5] and contributes to the formation of oxalic acid and the scaling of process circuits by calcium oxalate [7, 8].

Because of the above problem encountered in bleaching operation due to presence of HexA, some attempts to minimize the amount of HexA chemically and biologically in the pulp both in the kraft cook and in the subsequent bleaching have been made to produce a fully bleached pulp with high brightness stability with a low HexA content is crucial. Low HexA content is likely to provide a cleaner production opportunity and to be more effective due to lesser consumption of bleach chemicals and pollutant. Some efforts have been conducted to reduce HexA content in kraft pulp by inserting acidic treatment stages at high temperature without bleaching chemicals between cooking and bleaching [5] and using electrophilic bleaching chemicals such as chlorine based chemicals, ozone [9] and peracetic acid [10]. These chemical treatments have some limitations related with environmental issue such as formation of chloro-organic compounds that have harmful environmental effects [10] and loss of

Bio bleaching technologies, that employing enzymatic bleaching technique in the process has shown immense potential in minimizing the use of bleaching chemicals. Currently, the most important application of enzyme is in the pre-bleaching of kraft pulp. Xylanase (EC 3.2.1.8) and laccase (EC 1.10.3.2) have found to be the most effective enzymes for that purpose. Enzyme provides a very simple and quite effective way for industry without interfering with the existing process [12, 13]. Enzyme also offers a simple approach that allows for a higher brightness ceiling to be reached.

Xylanases hydrolyzes re-precipitated xylan on fiber surface selectively [14, 15, 16] and therefore enhance the accessibility of lignin in wood fibers to bleaching chemicals such as chlorine dioxide (ClO₂) and can reduce the required amount of bleaching chemicals required to produce pulps of desired brightness value [17]. Laccase play an important role in degrading the lignin that reduces the kappa number and enhance the bleaching of kraft pulp when they are used in the presence of laccase mediator system (LMS) such as 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonate (ABTS) and 1-hydroxybenzotriazole (1-HBT) [12].

Recent reports show that application of xylanase and laccase separately or in sequence in pulp bleaching can also reduce the content of HexA [13, 15, 16, 18, 19]. Some limitations of using these enzymes in pulp bleaching were pulp yield loss of up to 1% based on dry pulp because excessive hydrolysis of xylan by xylanase that not associated with residual lignin [20, 21]; harmfull effect of the laccase mediator system for lignin destruction [12] that needs to be solved. High xylan content is important, in term of both its contribution to pulp yield and its ability to improve pulp strength such as tensile properties. The need of enzyme with more efficient and high selectivity to
remove HexA from kraft pulp is a critical problem. Hence, there is an immediate need to examine enzymes for more efficient and direct enzymatic removal of HexA from kraft pulp. When these new proposed enzymes were used for HexA removal, there are some benefits which can be offer such as:

1. When HexA is selectively removed from the pulp using hexenuronic acid groups releasing enzymes, a better quality of pulp will be obtained, such as improvement of brightness stability and pulp viscosity,

2. By enzymatically removing the carboxylic groups of the hemicelluloses from the cellulose pulps, both the surface charge and the metal-ion content of the pulp can be changed,

3. When specific enzyme is used for HexA removal, the metal binding uronic acid groups can be removed which will decrease the amount of the metal-ions in the pulp. Hence the use of complexing agents (for instance ethylene diamine tetraacetic acid, EDTA or diethylene triamine penta acetic acid, DTPA) prior to Totally Chlorine Free (TCF) bleaching can be reduced or totally omitted,

4. The enzymatic removal of glucuronic acid groups can be used to improve the production of certain pulps, such as metal-free pulps or pulps with very low amounts of carboxyl groups [22].

However, information about enzyme that enable for direct removal of HexA from kraft pulp is still limited. In our study, HexA degrading enzymes were studied further and prepared using two Paenibacillus species. These Paenibacillus has different enzyme systems that play role to degrade ΔX3. First enzyme was α-Glucuronidase (EC 3.2.1.139) which prepared from Paenibacillus curdlanolyticus B-6. P. curdlanolyticus B-6 is an organism that can efficiently degrade xylan polymers substituted to varying
degrees with GlcA, MeGlcA, acetyl, feruloyl, or p-coumaroyl side-chain groups through a multienzyme complex of cellulases and hemicellulases [23]. α-Glucuronidase enzyme hydrolyzes the α-1,2 glycosidic bond between α-D-glucuronic acid (GlcA) or its 4-O-methyl ether (MeGlcA) and D-xylose residues of xylooligosaccharides (aldouronic acids). The majority of α-glucuronidases that hydrolyze these bonds are located in glycoside hydrolase family-67 (GH67) [24, 25]. These enzymes, remove uronic acid only from glucuronoxylooligosaccharides and not from glucuronoxylan. α-Glucuronidase might be applicable for the direct removal of HexA groups from xylooligosaccharides because of the similar structures between HexA and MeGlcA. In particular, there are no reports on whether α-glucuronidase can remove HexA from ΔX3. Therefore, it is important to characterize the α-glucuronidase from Paenibacillus strains that degrade ΔX3.

Next enzyme was HexA degrading enzyme which prepared from Paenibacillus sp. strain 07. Paenibacillus sp. strain is a soil bacterium that has ability to utilize ΔX3 as a carbon source [26]. Winyasuk et al. [2012] stated that crude intracellular fraction has an enzyme that capable to hydrolyze the first xylosidic linkages from the reducing-end side of ΔX3 and produced hexenuronosyl-xylobiose (ΔX2) and xylose [26]. This enzyme might belong to xylanase GH 8 [27]. The resulted ΔX2 then hydrolyzed further by HexA degrading enzyme and β-xylosidase to produce HexA and xylose as final products. Meanwhile, there was no report on whether extracellular fraction from this species can remove HexA from ΔX3. Therefore, characterization of extracellular enzyme produced from this strain is important to study further.
1.2 Objectives

In this dissertation, we study about hexuronosyl xylotriose degrading enzyme from *Paenibacillus* species. The objectives of this study are:

1. To determine direct removal of HexA from xylooligosaccharides using the GH67 α-glucuronidase from *Paenibacillus curdlanolyticus* B-6, an organism that can efficiently degrade xylan polymers substituted to varying degrees with GlcA, MeGlcA, acetyl, feruloyl, or p-coumaroyl side-chain groups through a multienzyme complex of cellulases and hemicellulases,

2. To characterize HexA degrading enzyme in the intracellular fraction and extracellular fraction from *Paenibacillus* sp. strain 07, a soil organism that able to utilize ΔX3 as a carbon source

Achievements of these objectives in these studies will provide a new insight into HexA removal from kraft pulp and may contribute to the reduced consumption of active bleaching chemicals during bleaching process. Moreover, some specific objectives of this study will be explained further in each chapter of this dissertation.

1.3 Structure and outline of this dissertation

Presence of HexA has significant role in bleaching process related with its impact to quality of bleached pulp. Some attempts to remove HexA were carried out using chemical treatment but some problems also encountered. Using of enzyme (bio bleaching) show a promising technology for HexA removal. Unfortunately information about HexA degrading enzyme is still limited. One of the studies has been conducted by Winyasuk et al. [2012], results showed that *Paenibacillus* species has an enzyme system in the intracellular fraction that able to remove HexA side chain from xylan backbone,
but there is some issues still remain related with the degradation mechanisms of HexA and what enzyme play role for HexA removal [26]. In this study we try to study further about directly remove HexA using recombinant and native enzyme from two kinds of Paenibacillus species to explain more about the degradation mechanisms. First of all, to study further about the degradation mechanism and possibility of using enzyme for HexA removal, hexenuronosyl xylotriose (ΔX3) was prepared from alkaline and enzymatically treated eucalyptus kraft pulp and use as a model substrate.

Regarding to that issues we try to study about the possibility of using GH67 α-glucuronidase (AguA) to remove HexA. These enzymes remove uronic acid only from glucuronoxylooligosaccharides and not from glucuronoxylan. α-Glucuronidase from P. curdlanolyticus B-6 (called B6) was used as a model in this study. α-Glucuronidase might be applicable for the direct removal of HexA groups from xylooligosaccharides because of the similar structures between HexA and MeGlcA. This study will be discusses further in the chapter 2.

In the next chapter, enzyme system in Paenibactillus sp. strain 07 (called 07) for HexA removal was examined further. The first step was to characterize further about ΔX3 degradation using crude intracellular and extracellular enzyme fraction from 07. The result of this study will be explained in the chapter 3. General conclusions are remarked in Chapter 4.
1.4 Literature review of hexenuronic acid (HexA) and current technology for HexA removal from kraft pulp

1.4.1 Hemicelluloses

Native hemicelluloses in hardwood and softwood are comprised of variety of five or six carbon cyclic sugar units such as glucose, mannose, galactose, xylose, arabinose, 4-\(O\)-methyl-glucuronic acid, and galacturonic acid residues. Some hardwoods contain trace amounts of rhamnose. They exhibit lower crystallinity, molecular weight and degree of polymerization than cellulose and some are branched. They are intimately associated with cellulose and appear to contribute as a structural component in the plant. Hemicelluloses are soluble in alkali and easily hydrolyzed by acids. These characteristics make this polymer more susceptible to chemical degradation in acidic and alkaline environment. Hardwoods contain greater amounts of glucuronoxylans, whereas softwoods are predominately composed of glucomannans. In addition to monosaccharide’s moieties, this polymer also contains uronic-acid type structures and acetyl groups. The uronic acid-type groups are almost exclusively 4-\(O\)-methyl-glucuronic acid, with trace amount of glucuronic and galacturonic acid.

1.4.2 Origins of Hexenuronic acid in pulps

In the early 1950s, the 4-\(O\)-methyl-glucuronic acid unit or 2-\(O\)-(4-methyl-\(\alpha\)-D-glucopyranosyluronic acid), was found to be the main acidic constituent of xylan in both hardwoods and softwoods. This finding led to intensive investigations into the alkaline degradation of 4-\(O\)-methyl-glucuronoxylan, which is xylan that containing 4-\(O\)-methyl-glucuronic acid groups.
In 1963, Clayton proposed that during kraft pulping 4-O-methyl-glucuronic acid was converted to hexenuronic acid (HexA) by the β-elimination of the 4-O-methoxyl group, after the loss of the hydrogen atom attached to the fifth position of the glucuronic acid residue (Fig. 1.1) [2]. Then, in 1977, Johansson and Samuelson verified further this reaction using 2-O-(4-O-methyl-α-D-glucopyranosyluronic acid)-D-xylitol as a model compound [28]. When this compound treated with sodium hydroxide at elevated temperatures; a new acidic substituent, 4-deoxy-β-L-threo-hex-4-enopyranosyluronic acid (hexenuronic acid, HexA) was formed. Teleman et al [1996] investigated further the occurrence of HexA group during kraft pulping using Nuclear magnetic resonance (NMR) spectroscopy and mass spectroscopy [2].

Based on some reviews, glucuronic acid groups are the precursor for HexA formation. Softwoods commonly contain 1.2-5.0% of these groups, whereas hardwoods contain slightly higher, about 2.0-6.0%. Hardwoods contain 20 to 25% glucuronoxylans as opposed to 6 to 12% for softwoods [1]. Li and Gellersted [1997] reported that typically 3-6 KN unit of unbleached hardwood pulp and 1-3 KN unit of an unbleached softwood pulp are due to HexA and not lignin [29]. The calculation was based on a molar basis using two different ways (permanganate oxidation of model compounds and selective elimination of HexA groups from a series of kraft pulp). Result showed that 10 µmol of HexA was corresponded to 0.84 – 0.86 kappa units.

1.4.3 Hexenuronic acid behavior during pulping

HexA is one of the main uronic acid constituents in the fractions of xylan found in kraft pulping and in bleaching liquors [2]. This group contains enol ether or unsaturated
carboxylic acid groups that will strongly influence the stability and reactivity of HexA. In general, these enol ether and unsaturated carboxylic acid groups are inert in the mild condition [8]. HexA has different behavior in alkaline and acid solution. In the strong alkaline solution, HexA decomposes slowly at high temperature, for example, hexenuronoxyllose (model compound of HexA), has a low decomposition rate constant about 0.52/h in 1M NaOH at 150°C. On the other hand, HexA is an acid labile; in a mild acid hydrolysis this group would yield furan derivatives such as 5-formyl-2-furan carboxylic acid (FF) and 2-furan carboxylic acid (FA) [2, 3].

Content of HexA in unbleached alkaline pulp are the results of two competitive processes: formation and degradation/dissolution of HexA [30, 31]. Some parameters in pulping process such as effective alkali (EA) concentration and alkaline consumption [30, 32, 33], cooking temperature [34], sulfidity, liquor-to-wood ratio and species being pulped affect the amount of HexA in pulps [31, 35, 36]. Pedroso & Carvalho [2003] stated HexA content in pulp is a complex result of the cooking parameters, that not only affect delignification rate but also other pulp characteristics that are relevant for the final product behavior [34].
Fig. 1.1 The conversion of 4-\textit{O}-methyl-D-glucuronoxylan to hexenuronoxylan during kraft pulping as suggested by Clayton in 1963 [Teleman et al. 1996] [2]
An increase in liquor alkalinity up to some critical concentration accelerates the conversion of uronic acid to HexA. But the subsequent increase in alkalinity causes the degradation of HexA. Shatalov and Helena [2004] showed that effective alkali concentration in the cooking liquor is only a controlling factor for HexA formation and degradation in alkaline pulping [33].

Daniel et al. [2003] showed HexA content in E. globulus pulps increased with increasing pulping time (degree of delignification) [31]. At the same kappa number (KN 14), HexA content decreased when active alkali (AA) increased from 17% to 24% and sulfidity increased from 15% to 28% respectively. Result also showed that HexA content decreased when pulping temperature rose from 150°C to 170°C and when liquor-to-wood ratio increased from 4.0 to 8.0.

Other study conducted by Pedroso & Carvalho [2003] using E. globulus kraft pulps showed that effective alkali (EA) and temperature were the main factors affecting the amounts of HexA in pulps [34]. Result showed effect of cooking temperature on HexA content depends on EA level. For lower level of EA (12.8%), an increment in cooking temperature raises the HexA content, the opposite result was observed for the upper EA values (17%). In this study, it was mentioned that effect of sulfidity charge seems to be significant for HexA content, this result was opposed to others finding.

Different behavior of HexA formation was shown in softwood and hardwood kraft pulping. In softwood, HexA is formed essentially during the initial of kraft pulping stages (namely heating-to-cooking temperature) [30]. At the final cooking temperature HexA content in pulp decreases continuously with time due to alkaline degradation and xylan dissolution. Some previous study showed that the rate of HexA degradation increases with the concentration of hydroxide and hydrogen sulfide anions. The increase
in pulping temperature leads to lower HexA contents in pulp [31]. Buchert et al [1995] showed that HexA degradation during kraft pulping of the pine wood (*Pinus sylvestris*) started only after 90 min of the process at 170°C [35]. Meanwhile, during hardwood pulping that carried out at less than 170°C, HexA content increased as pulping proceeded, and degradation of HexA was not observed during most of the pulping process [32]. These different behaviors of HexA content in two native woods might be to the milder pulping conditions that were used in the hardwoods than softwood [30].

In addition to the influence of process variables during pulping, HexA content in pulp also depends on the species being pulped. For example, hardwood pulps contain more HexA groups than softwood pulps even cooked in similar condition [5], because hardwoods contain more 4-\(O\)-methylglucuronoxylan than softwoods [1].

### 1.4.4 Hexenuronic acid behavior during bleaching process

HexA shows some harmful effects on the bleaching process because of its unsaturated structure [34]. HexA units contain conjugated carboxylic acid groups, which can react and consume some electrophilic bleaching chemicals such as chlorine dioxide and ozone, peroxyde (when combined with ozone bleaching) and peracetic acid in the subsequent bleaching stage. These bleaching chemicals react with the 'ene' functionality of HexA groups [35]. This means part of bleaching chemicals are not consumed during delignification process, that lead to higher bleaching chemical consumptions, increased production costs, and increased effluent emissions [37].

HexA also binds transition metal ions such as manganese (II) which are known to catalyze the degradation of hydrogen peroxyde during peroxyde bleaching [38]. This
metal binding leads to intensifying the use of expensive complexing agents in hydrogen peroxide bleaching such as EDTA.

The presence of HexA not only increases the difficulty of reaching a high degree of brightness, but also increases brightness reversion (yellowing). HexA groups, even in a small amount (<1% on pulp), reduce the brightness stability because of their high affinity for transition metal ions [39].

Unsaturated structure of HexA group contributes to overall unbleached pulp KN because they consume part of potassium permanganate used in this determination as well as lignin does [4]. Kappa number is a control parameter for estimating lignin content in pulps and to evaluate the efficiency of delignification in pulping and bleaching processes. Presence of these unsaturated constituents affects analysis results by giving higher amount of residual lignin in the pulp. These effects are more significant on hardwood than on softwood kraft pulps because hardwood contain more HexA for the same delignification degrees [30].

Li & Gellerstedt [1998] demonstrated HexA contributes about ⅓ of overall KN of unbleached birch pulp and about 60 to 70% of the measured KN of O2 delignified birch pulps [4]. Therefore, they also mention, overall the presence of HexA groups can contribute significantly to the measured value of kappa number: 3–6 kappa number units for hardwood kraft pulp and 1–3 kappa number units for softwood kraft pulps. Oxidative degradation of HexA with chlorine dioxide and ozone may also contribute to the formation and deposition of oxalic acid in the bleaching equipment [3].
1.4.5 Current technology for hexenuronic acid removal

1.4.5.1 Chemical treatment

Overall some technologies related with chemical treatment have been introduced to remove HexA groups from kraft pulps selectively. The technologies can be applied before bleaching process or become a part of the bleaching sequence.

A. Removal of hexenuronic acid groups before bleaching

Some technologies have been developed to remove HexA groups from kraft pulps selectively before bleaching process since HexA reacts with some bleaching chemicals. Most of the removal technology related with mild acid treatment (A stage). When this acid stage used for HexA removal, HexA concentration in pulp decreased significantly, the decreasing depends on the condition that used in the treatment such as temperature and reaction time.

Vuorinen and coworkers [1999] stated a mild acid treatment could selectively remove 90% of HexA in birch kraft pulp, when an A stage operated at 85 to 115°C between pH of 3 and 4 for 45 minutes to 10 hours [5]. Another study conducted by Jiang et al. [2006] showed approximately 60% of HexA groups in kraft pulp can be removed by acid hydrolysis at 90-95°C and pH 3 for 3h, or at 120 °C and pH 3.5-4.0 for 45 min [39]. Meanwhile, Andrew [2007] stated that approximately 95% of HexA in Eucalyptus pulp removed at temperature of 110°C and reaction time of 120 min. Any further increase in temperature and/or reaction time beyond this point had no significant impact on the residual amount of HexA in the pulp [40].

Besides that, acid treatment of kraft pulp has been of interest for various reasons such as:
1. An acid treatment before oxygen delignification, for example, could increase brightness by 2–6 points, improve the brightness stability of bleached kraft pulps, which is consistent with the general influence of uronic acids on the brightness stability of pulp and enhance the degree of delignification without excessive degradation of polysaccharides [8]. Andrew [2007] stated brightness reversion was found to decrease significantly as the temperature and reaction time of the A-stage increased [40].

2. At 110°C and 120 min incubation, kappa number of Eucalyptus pulp was reduced by up to 5 units. Therefore decrease in kappa number during acid hydrolysis was attributed largely to the decrease in the concentration of HexA and not to the loss of lignin [40].

3. Furthermore, acid treatment could be used in total chlorine free bleaching sequences to remove transition metals ions such as Fe and Mn from pulp to prevent catalytic decomposition reaction with H₂O₂ and O₂, which negatively affect pulp quality and impair some brightening reactions [41]. Acid treatment able to removes metal ions more effective than does a milder acid washing step. This result suggests that metal ions in kraft pulps are mainly bound to uronic, especially hexenuronic, acid groups.

4. Acid hydrolysis was also found to have no significant impact on most of the physical properties of the Eucalyptus bleached pulps. Properties such as burst, tensile, zero-span tensile, stretch, stiffness, air resistance and sheet density were either unaffected by the acid treatments, or in some cases, showed improvements after the acid treatments.

5. The removal of hexenuronic acid groups in hardwood kraft pulps prior to bleaching can provide substantial savings in chlorine dioxide [11]. Because softwood kraft
pulps contain fewer hexenuronic acid groups, the savings are less substantial for softwood pulps.

B. Removal of hexenuronic acid groups in bleaching sequences

HexA removal in bleaching sequences can be achieved using some electrophilic chemical bleaching such as chlorine dioxide, ozone, oxygen delignification and peracid acids. Buchert et al [1995] stated HexA level on Pinus sylvestris was impacted by chlorine dioxide (ClO$_2$) treatment in bleaching sequence [35]. Amount of HexA in the O$_2$-delignified pulp was almost completely eradicated after D2-stage. Amount of eliminated HexA by ClO$_2$ bleach sequence vary widely, depending on entering level of HexA and KN, and total charge of ClO$_2$. Vuorinen et al. [1997] noted that HexA are consumed upon treatment with ClO$_2$ under acidic conditions [38]. These reaction lead to formation of organochlorine compounds, specifically chlorinated dicarboxylic acid structures.

Attempt to remove HexA also conducted at oxygen delignification step. Some researchers stated there was no significant difference between unbleached and oxygen delignified pulps in HexA content, due to unreactivity of HexA to oxygen [4, 5, 35, 40].

Ozone is the most powerful chlorine-free oxidant available. When an ozone (Z) stage bleaching treatment was carried out on an oxygen-delignified eucalypt pulp, HexA and lignin are attacked and removed from the pulp, but cellulose depolymerization also occurred [42]. If the lignin removed and HexA was left, the amount of depolymerization was even more severe. Reaction between HexA and ozone is the origin of some cellulose depolymerization. The proposed mechanism was ozone will react with carbon-carbon double bond of HexA generates radicals that leads to the polymerization
The way to minimize cellulose degradation during ozone delignification is to remove or to avoid the presence of carbon-carbon double bonds in the medium as much as possible. The approach would be to eliminate HexA before ozone treatment, such as using acidolysis stage (A) in the conventional ECF [11].

Petit-Breuilh et al. [2004] conducted a study related with using peracid acids, peroxymonosulphuric (Ps) acid, to remove HexA present in unbleached eucalyptus kraft pulps [37]. Pulp were treated at different temperature from 20°C to 110 °C with Ps concentration ranging from 0.2-1.0 %. Experimental results show that Ps effectively removed both HexA and lignin, even under mild conditions. Selectivity towards HexA increased at higher temperature and lower Ps loads. However, at lower temperatures and higher Ps concentrations, cellulose may also be attacked during treatment, leading to significant reductions in intrinsic viscosity.

### 1.4.5.2 Biological treatment for HexA removal

Biobleaching technologies, employing enzymatic bleaching techniques, is now considered to one of the preferred routes, primarily because of advantages offered over conventional chemical bleaching. Major advantage is reduction of toxic chemicals, adsorbable organic halide (AOX) in the discharge effluents by reducing the requirement of chlorine bleaching and improved pulp quality gain in brightness [12]. The use of enzymes has emerged as a very promising choice not only for implementing clean technology processes, but also for developing novel, high-added value product [16]. Most widely used enzyme for HexA removal was xylanase and laccase. An attempt to use other enzyme such as lipase in sequence with xylase was also observed [21].
enzyme was used separately or in sequence to remove HexA in pulp.

A. Xylanase for HexA removal

Xylanase has been long recognized as an effective bleaching pre-treatment. Endo-1,4-β-D-xylanases (E.C 3.2.1.8) depolymerase xylan by random hydrolysis of xylan backbone. Early xylanase treatments were made at mildly acidic pH and low temperatures using low concentration of enzymes [15]. The efforts of most enzyme producers towards improvement resulted in better products which are active within neutral or alkaline pH range. The primary purpose of xylanase pre-treatment in pulp bleaching is to improve pulp bleachability, basically assumed as a capacity to brightness development in the subsequent chemical bleaching stages (bleach boosting) [14].

The beneficial effect of xylanase on kraft pulp bleaching is selective hydrolysis of xylan re-precipitated on the fiber surface and therefore improving fiber permeability to bleaching reagents [14, 15, 16] and hydrolysis degradation products or to the increased extractability of lignin-carbohydrate complexes (LCC) and carbohydrate-derived chromophores causing the change in pulp brightness, that facilitates the pulp delignification during subsequent chemical bleaching steps [14]. Application of xylanases as bleach boosting agents has known to save chemical costs for industry without interfering with the existing process [12, 13]. Their use also allows a notable reduction in the dose of bleaching chemicals and minimizes pollutants production [43]. Major limitation of xylanase application was excessive hydrolysis of xylans that not associated with residual lignin [21].
A very innovative aspect related with the use of xylanase is that these enzymes can reduce the content of HexA of the pulps [12, 13, 14, 16, 19], which is remarkable secondary effect. Xylanases hydrolyses xylans on fiber surfaces and such xylan contains HexA, these compounds may be removed by enzymatic treatment with xylanases [18]. Some xylanases from different families (GH 10, 11 and 5) has been evaluated for HexA removal [13, 15, 44]. Each family of xylanase gave different behavior when applied for HexA removal, this difference depend on several factor such as the xylan polymer nature, pulping process, the chain length, xylan content of fibres, type and accessibility of xylan and the presence of substituents as well as on the amount of xylans [13, 15, 44].

Shatalov and Pereira [2009] applied commercial xylanase for biobleaching some chemical pulps. Unbleached industrial eucalypt (E. globulus L.) kraft pulp, three non-woody (giant reed or Arundo donax L.) organosolv pulps were used in bio-bleaching experiment [14]. The pulps were treated with commercial xylanase preparations endo-1,4-β-xylanase; E.C 3.2.1.8) and the change in pulp properties was defined in comparison with enzyme-free samples (control). Result showed that xylanase treatment caused a direct pulp brightening observed with all the tested pulps. A good agreement can be observed between change in brightness and HexA removal. There was a strong positive correlation between gain in brightness and HexA loss during xylanase treatment of different chemical pulp, thereby pointing to HexA as an important factor for direct pulp brightening. To define contribution of HexA-cause brightening to total xylanase-assisted brightness improvement, the selective removal of HexA from eucalypt kraft pulp was performed before xylanase treatment. Then the HexA-free pulp was then treated by xylanase pre-treatment and examined on change in
optical properties before and after enzymatic treatment. Result showed the preliminary HexA removal can increase the pulp brightness and the following xylanase treatment did not affect pulp brightness. Based on this result direct brightening effect observed during xylanase treatment of chemical pulps is primarily caused by the enzymatic removal of HexA.

A multi-stage hydrogen peroxide bleaching was chosen to assess the effect of HexA on xylanase bleach boosting. This chemical is a fairly effective chlorine-free oxidative chemical which is widely used as an integral part of all modern TCF bleaching sequences. Result showed single-step of hydrogen peroxide bleaching causes additional brightness improvement of enzymatically pre-treated pulp up to 3% ISO in comparison with control, as a result of xylanase bleach boosting effect that accompanied by additional loss of HexA and lignin.

In 2010, Valls et al. evaluated the effects produced by new xylanases from different families (GH10, 11 and 5) and bacterial strains (Bacillus sp. BP-7 and Paenibacillus barcinonensis) on HexA content and pulp bleachability of eucalyptus kraft pulp [15]. Results showed GH11 was the most efficient in increasing delignification, pulp bleachability, brightness and HexA removal comparing with other xylanases. Same result also obtained by Esteghlalian et al. [2008] [17]. Result showed that family 11 xylanases appear to be in general more effective than family 10 xylanases in prebleaching application intended to reduce the use of pulp bleaching chemicals. An outstanding result is that GH5 produced a similar effect in increasing delignification and brightness than GH11, but cannot increase HexA removal. The results obtained for GH5 need further studies to test application of the enzyme at
different condition. On the other hand, GH10 increased exclusively the HexA removal, but not enhanced pulp bleachability as much as GH11 [15].

Recent study has been conducted to evaluate the effects of five xylanases including supernatant cultures (enzyme cocktail with contains GH10 as its predominant component) and recombinant enzymes from *Paenibacillus barcinonensis* (GH10, Xyn30D, Xyn30Dcat from GH 30, and GH11) on sisal fibers [13]. Different result was obtained; GH10 was the most efficient xylanase for lignin and HexA removal from sisal fibers. When enzyme cocktail from *P. barcinonensis* (Pba crude xylanase) used, similar results were obtained.

Xylanases belonging to GH30 were more efficient when applied as a single catalytic domain than Xyn30Dcat, because Xyn30D is a modular xylanase containing the carbohydrate-binding module 35 (CBM-35) that has greater size (62 kDa) than Xyn30Dcat (47 kDa). The accessory modules may decrease the bleaching efficiency of an enzyme, because of the difficulties a larger molecule encounters in accessing the lignin trapped in pulp fibers. The lower bleaching capacity of Xyn30D could also be caused by its glucuronoxylan binding ability [45] as this might decrease the free diffusion of the enzyme between fibers. By contrast, GH11 did not have any significant effects on pulp properties. The release of sugar in the X stage effluents correlates with the effectiveness of the enzymes tested. The effectiveness of xylanase depends on the xylan content of fibers and on the type and accessibility of these xylans.

Results also showed that all xylanases treatments reduced HexA content. The best results were obtained with GH10 and Pba crude xylanase, which reduced the total HexA of the pulp samples by around 25%. Xyn30D and Xyn30Dcat treatments led to HexA reduction of between 11 and 14%, which contrasted with previous findings that
report a smaller effect of the xylanase belonging to GH30 in HexA removal from eucalyptus fiber [15].

Study conducted by Gallardo et al. [2010] showed that GH5 was effective in boosting and delignification and bleaching, when applied to *E. globules* kraft pulp, as a decrease in KN and increase of brightness were found [44]. The effect of GH5 was less pronounced than GH11. However, when the two enzymes were applied together, a synergistic effect was found in increase of brightness over without enzyme treatment. The effect on these enzymes on HexA showed that xylanase-treated pulps showed a decreased HexA content, indicating that xylanase contribute to the removal of this compound. GH5 had a less pronounced effect than GH11 for HexA removal.

**B. Lipase for HexA removal**

Nguyen et al. [2008] stated that commercial lipase (Amano) and xylanase pre-treatment resulted in bleached pulp with lower kappa number and hexenuronic acid content compared to those obtained from control experiments [21]. Lipases from Amano (EC. 3.1.1.3) contain a significant feruloyl esterase activity and some other accessory enzyme activity. The reduction of kappa number and hexenuronic acid content after lipase treatment of different kraft pulps suggest that feruloyl esterase and/or other accessory enzymes may be associated with this direct bleaching effect.

In this study, lipaseA treatments led to a reduction of hexenuronic acid of between 13% and 24.4% in the unbleached hardwood kraft pulp (UBHW), oxygen delignified hardwood kraft pulp (O₂HW), unbleached softwood kraft pulp (UBSW) and oxygen delignified softwood kraft pulp (O₂SW). When the reduction in HexA is calculated based on its percentage of kappa number, it was found that the reduction in
HexA represents 26%, 36%, 51% and 27% of the kappa number removal in the UBHW, O₂HW, UBSW and O₂SW respectively.

The presence of a complex of HexA and lignin has been proposed, and such a structure is possibly a major reason for the recalcitrance of residual lignin. Based on this result that lipaseA specifically degraded HexA and in consequence removed the lignin attached to these acids. These results also substantiate the theory that HexA are a site for the formation of lignin-carbohydrate complexes (LCCs).

A pulp treated with lipaseA also demonstrated a better bleach ability than one treated with commercial xylanase. Bleaching lipaseA pretreated UBHW in a D₀E sequence resulted in a chlorine dioxide saving up to 38% compared to the control. This saving is much more significant than obtained with xylanase pre-bleaching. Bleaching selectivity is considered as one of the important criteria for a superior bleaching enzyme. Based on amount of the sugar released in the filtrates after enzymatic treatment, the results showed that lipaseA only released a small amount of xylose. It is indicated that lipaseA highly specific for removing xyloses associated with either residual lignin and/or hexenuronic acids.

C. Laccase for HexA removal
Laccase (EC 1.10.3.2) is a blue copper enzyme with broad substrate specificity that catalyzed the oxidation of phenol, anilines and aromatic thiols with the concomitants four electron reduction of O₂ to H₂O [46]. A combination between laccase with a chemical mediator called “laccase mediator system” (LMS) have shown positive effects than the use of xylanase alone for bio bleaching when use for pulp delignification such as reduction in kappa number, improvement in brightness, structural modification of the
residual fiber lignin, and removal extractives such as sterols in pulps [16, 18, 19]. Previous study conducted by Valls and Roncero [2010b] showed the effect of HexA content reduction in eucalyptus kraft pulp by a laccase-HBT system and found it to be strongly boosted by xylanase pretreatment [18]. Effectivity of laccase for HexA removal depends on source of laccase, application condition, and type of mediator [16, 19]. Major hurdles for industrial implementation of LMS-based processes are concern about the potential toxicity of synthetic mediator, cost of mediators [12, 46] and produce of hydroxyl radical which can lead to cellulose oxidation and consequent decrease in the degree of polymerization, manifested a viscosity decrease [16, 18, 46].

Valls et al [2010b] examined evolution of HexA and lignin during a laccase-1-hydroxybenzotriazole (1-HBT) treatment (an L stage) in the presence and absence of a novel xylanase-based pretreatment (an X stage) [18]. HexA content decreased when laccase-HBT treatment was applied, the greatest effect was observed at high levels of the operating conditions. This result suggests that laccase-HBT system destroys HexA. HexA was oxidized by laccase-HBT system similarly to electrophilic bleaching agents such chlorine dioxide, ozone and peracids. Viscosity was decreased when high level of laccase used. The laccase-mediator treatment can degrade cellulose via the formation of carbonyl groups capable breaking cellulose chains by β-elimination in an alkaline medium. When the pulp was pretreated with xylanase, kappa number reduction increases significantly about 11% higher than only laccase treatment. Other effects were pulp samples were brighter and HexA were removed more efficiently.

Aracri and Vidal [2011] applied laccase (L stage) and xylanase (X stage) for HexA and lignin removal from sisal fiber [16]. It was found kappa number reduction caused by L stage was higher in the xylanase pre-treated pulp than in the initial pulp.
This suggests that the X stage increased the accessibility of the laccase-Violuric acid (VA) system into pulp fibers, thereby facilitating removal of lignin and HexA contributing to KN, which is consistent with the bleach boosting effect of xylanase treatments previously observed by other authors. This L stage exhibited a high efficiency in raising pulp brightness in all stages especially when xylanase pretreatment applied. There was a viscosity reduction in the oxygen delignification stage when VA, probably the mediators oxidized carbohydrate chains in cellulose to carbonyl groups during the L stage thus making the pulp vulnerable to degradation by the strong alkaline medium used in the bleaching stage.

Xylanase treatment successfully removed a substantial fraction of HexA in the initial pulp (27%) by releasing xylan chains from fiber surfaces. Interestingly, laccase reduced the HexA content in the control treatment especially at higher enzyme dose. HexA reduction was higher in the laccase-VA treated pulp, to the same extent as that produced by xylanase, which suggest that this system can destroy HexA by oxidizing their double bonds similarly to electrophilic bleaching chemicals. They also found that removal of HexA in the laccase-LMS system is depend of the mediator type, they said laccase-sinapyl aldehyde (SLD) system exhibited no reduction in the HexA content.

Based on the effluent quality such as chemical oxygen demands (COD) and color values, xylanase treatment produced a high COD accounting for more than 40% of total value for the sequences including an X stage. This can be ascribed to substantial carbohydrate degradation and lignin removal of this stage, which testifies to the efficiency of enzyme. This contribution is the greatest disadvantage of using xylanase in bleaching process. COD after L stage was markedly higher than after chelating agents and oxygen stages, this was mainly a result of the presence of sodium tartrate buffer and
use of laccase. The effluent also contains mediator and degradation products of the mediator and lignin. The combined COD for all stages was significantly higher when a xylanase treatment was applied than laccase-mediator only. The same results also found for the effluent color, the color after L stage increase compared with control due to increasing concentration of chromophoric resulting from oxidator and/or degradation of lignin and the mediators. Similar to COD, the color of effluent was higher after xylanase treatment than from L stage.

In this study, the treatment with xylanase and laccase in the absence of mediator led to low toxicity values, the increase was especially marked with laccase-SLD treatment. The effluent toxicity using this treatment was six times higher than laccase-VA treatment. Toxicity was due mainly to the presence of pulp in the laccase-VA treatment and to oxidation of the mediator by laccase in the laccase-SLD treatment.

Cadena et al [2011] applied some fungal laccase–mediator combinations followed by physical and chemical characterization of the flax soda pulps to obtain a thorough understanding of the laccase/mediator effects on hexenuronic acid (HexA) removal and the coupling of mediator onto pulps for fiber functionalization [19]. They found efficiency of HexA removal stronger than effect of acidic hydrolysis (A-stage) and hot chlorine dioxide bleaching stage when laccase form *Trametes villosa* and lauryl gallate (LG) was used. Decreasing of HexA concentration should be due to direct oxidative elimination of the easily accessible HexA located on the outer parts of the fiber since laccase cannot penetrate deep into the pulp fiber wall. When mediator used, HexA content reduce further showing that oxidized form of mediator could penetrate deeper into the fiber wall and attack HexA located in these parts.
In this study, they found that there was an increasing of kappa number in unbleached and bleached pulp after treated by laccase with lauryl gallate and p-coumaric acid, because the aromatic/phenolic structure of two mediators, these should be prone to oxidation by acidic permanganate thus contributing to kappa number.

1.5 References


38. Vuorinen, T., Fagerström, P., Räsänen, E., Vikkula, A. [1997]. Selective hydrolysis of hexenuronic acid groups open new possibilities for development of


Chapter 2

The GH67 α-glucuronidase of *Paenibacillus curdlanolyticus* B-6 removes hexenuronic acid groups and facilitates biodegradation of the model xylooligosaccharide hexenuronosyl xylotriose

Overview

4-O-Methylglucuronic acid (MeGlcA) side groups attached to the xylan backbone through α-1,2 linkages are converted to hexenuronic acid (HexA) during alkaline pulping. α-Glucuronidase (EC 3.2.1.139) hydrolyzes 1,2-linked MeGlcA from xylooligosaccharides. To determine whether α-glucuronidase can also hydrolyze HexA-decorated xylooligosaccharides, a gene encoding α-glucuronidase (AguA) was cloned from *Paenibacillus curdlanolyticus* B-6. The purified protein degraded hexenuronosyl xylotriose (ΔX3), a model substrate prepared from kraft pulp. AguA released xylotriose and HexA from ΔX3, but the Vmax and kcat values for ΔX3 were lower than those for MeGlcA, indicating that HexA side groups may affect the hydrolytic activity. To explore the potential for biological bleaching, ΔX3 degradation was performed using intracellular extract from *P. curdlanolyticus* B-6. The intracellular extract, with synergistic α-glucuronidase and β-xylosidase activities, degraded ΔX3 to xylose and HexA. These results indicate that α-glucuronidase can be used to remove HexA from ΔX3 derived from pulp, reducing the need for chemical treatments in the pulping process.
2.1 Introduction

During alkaline pulping of wood chips, 4-O-methyl-D-glucuronic acid (MeGlcA) groups attached exclusively at α-1,2 linkages to the xylan backbone are about 75–90% degraded. The residual MeGlcA groups are converted to unsaturated 4-deoxy-β-L-threo-hex-4-enopyranosyluronic acid (hexenuronic acid or HexA) groups by β-elimination of methanol directly or via the intermediate product 4-O-methyliduronic acid [1, 2] (Fig. 1). HexA is a major uronic acid substituent of both industrial kraft pulp and some unconventional alkali-based organosolv pulps, accounting for 83–88% of total uronic acids [3]. The rate of formation of HexA depends on operating variables of the kraft process, including cooking time [4], temperature [5], and effective alkali concentration [6, 7]. HexA groups are significant because of their role in the bleaching process and their influence on the final pulp properties. HexA contributes to kappa number [8], increases the consumption of bleaching agents [9], retains metal ions, increases brightness reversion [10], and contributes to the formation of oxalic acid. There have been attempts to reduce the HexA content of pulp by inserting acid stages between cooking and bleaching [9], using an electrophilic oxidant such as elemental chlorine or chlorine dioxide, or using ozonation or a peracid treatment [11]. Enzymatic methods have also shown promise. Specifically, the use of xylanases and laccases in pulp-bleaching sequences has proved effective for removing HexA [12-14], indicating that enzymatic bleaching is a promising technology for the pulp and paper industry. However, these enzymes have low efficiency: only about 30% of HexA was removed from eucalyptus pulp treated with laccase and xylanase [14]. Furthermore, the addition of hydroxybenzotriazole is necessary for the removal of HexA by xylanases and laccases [12]. Therefore, more efficient and direct enzymatic bleaching
technologies are required for the pulp and paper industry.

α-Glucuronidase (EC 3.2.1.139) hydrolyzes the α-1,2 glycosidic bond between α-d-glucuronic acid (GlcA) or its 4-O-methyl ether (MeGlcA) and D-xylose residues of xylooligosaccharides (aldouronic acids). The majority of α-glucuronidases that hydrolyze these bonds are located in glycoside hydrolase family-67 (GH67) [15, 16]. These enzymes, however, remove uronic acid only from glucuronoxylooligosaccharides and not from glucuronoxylan. α-Glucuronidase might be applicable for the direct removal of HexA groups from xylooligosaccharides because of the similar structures of HexA and MeGlcA (Fig. 2.1). It was recently reported that a xylanolytic Paenibacillus strain could degrade hexenuronosyl xylotriose (ΔX3) using a combination of intracellular and extracellular enzymes such as xylanases and β-xylosidase; however, it is still unclear how these enzymes cooperate to degrade ΔX3 [17]. In particular, there are no reports on whether α-glucuronidase can remove HexA from ΔX3. Therefore, it is important to characterize the α-glucuronidase from Paenibacillus strains that degrade ΔX3. To determine whether α-glucuronidase can remove HexA, we prepared ΔX3 from alkaline and enzymatically treated eucalyptus kraft pulp for use as a model substrate. Here we report the direct removal of HexA from ΔX3 using the GH67 α-glucuronidase from *Paenibacillus curdlanolyticus* B-6. This organism has been well characterized for xylanolytic enzymes and can efficiently degrade xylan polymers substituted to varying degrees with GlcA, MeGlcA, acetyl, feruloyl, or p-coumaroyl side-chain groups through a multienzyme complex of cellulases and hemicellulases [18, 19]. α-Glucuronidase from *P. curdlanolyticus* B-6 may improve the brightness stability of pulp through enzymatic removal of HexA.
4-\textit{O}-methyl-glucuronoxylan

\[ \text{\textit{O}-methyl-glucuronoxylan} \]

\[ \alpha\text{-glucuronidase (GH4, GH67, 115)} \]

Hexenuronoxyl xylan

\[ \text{Hexenuronoxyl xylan} \]

Fig. 2.1 Structures of 4-\textit{O}-methyl-glucuronoxylan and hexenuronoxyl xylan
2.2 Materials and Methods

2.2.1 Bacterial strains and plasmids

*Paenibacillus curdlanolyticus* B-6 has been deposited with the BIOTEC Culture Collection of the National Center for Genetic Engineering and Biotechnology, Thailand (BCC No. 11175). *P. curdlanolyticus* B-6 was grown on Berg’s mineral salt medium, pH 7.0 [18, 20], containing 2 g of NaNO₃, 0.5 g of K₂HPO₄, 0.2 g of MgSO₄·7H₂O, 0.02 g of MnSO₄·H₂O, 0.02 g of FeSO₄·7H₂O, and 0.02 g of CaCl₂·2H₂O and supplemented with 5 g of birchwood xylan (Sigma-Aldrich, St. Louis, MO, USA) per liter of distilled water. Chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan).

*Escherichia coli* strains JM109, HST 08, and BL21 (DE3) (Takara Bio, Shiga, Japan) and plasmids pTAC-1 (DynaExpress TA PCR Cloning Kit, Tokyo, Japan) and pET22b (Merck KGaA, Darmstadt, Germany) were used for cloning and expression. *E. coli* cells were grown at 37°C in Luria–Bertani (LB) medium containing ampicillin (100 μg/mL).

2.2.2 Cloning and sequencing of the α-glucuronidase gene from *P. curdlanolyticus* B-6

Genomic DNA was prepared by phenol/chloroform extraction [19] and plasmid DNA was prepared using a QIAprep Spin Miniprep Kit in accordance with the manufacturer’s protocol (Qiagen, Frederick, MD, USA). The oligonucleotide primers used in this research are listed in Table 1. After comparing the amino acid sequences of more than five GH67 α-glucuronidases from *Paenibacillus*, we designed two degenerate primers (Table 1) based on two highly-conserved amino acid sequences (consensus sequences of DGSIERGYAG and SGKTIVIQHIY). To amplify the corresponding region from *P. curdlanolyticus* B-6 genomic DNA, polymerase chain reaction (PCR) was performed
with *Ex Taq* polymerase (Takara Bio) under standard conditions according to the manufacturer’s instructions. The purified PCR products were ligated with pTAC-1 and both strands were sequenced using M13 primers. Homology analysis through BLAST confirmed that the amplified region was a fragment of an α-glucuronidase gene. Four gene-specific primers (Table 1) were designed to perform genome-walking PCR using the Universal GenomeWalker 2.0 kit (Takara Bio) according to the manufacturer’s instructions. The purified products of the nested PCR were cloned into pTAC-1 and sequenced. Sequence assembly was performed with GENETYX ver.12 software (GENETYX CORPORATION, Tokyo, Japan). Nucleotide and amino acid sequences were analyzed with the blastn and blastp programs, respectively (https://blast.ncbi.nlm.nih.gov/Blast). The GH67 α-glucuronidase gene from *P. curdlanolyticus B-6* was named *aguA*.

### 2.2.3 Construction of the *AguA* expression vector

Specific primers (Table 2.1) were designed to amplify the open reading frame of the *aguA* gene and to provide restriction sites for generating fragments for ligation and cloning. A 2,070-bp fragment of the *aguA* gene was produced by PCR using *Ex Taq* polymerase (Takara Bio) under the following conditions: one cycle of 1 min at 98°C; 30 cycles of 10 s at 98°C, 1 min at 60°C, and extension for 2 min at 68°C; and an additional extension for 10 min at 72°C. The PCR products were purified by agarose gel electrophoresis, digested with *Nco*I plus *Xho*I, and cloned into the vector pET22b digested with *Nco*I and *Xho*I.
Table 2.1 Oligonucleotide primers used for cloning of aguA

<table>
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<tr>
<th>Primer</th>
<th>Sequence (5′ → 3′)</th>
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<tr>
<td>Degenerate primers&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>AguMF1</td>
<td>GAY GGN WSN ATH GAR MGN GGN TAY GCN GGN</td>
</tr>
<tr>
<td>AguMR1</td>
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<td>GWF2</td>
<td>CTCGAAGGGCTGATCGAGCCAGAGACA</td>
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<tr>
<td>Expression primers&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>AguA_pET22F</td>
<td>CATG CCATG GGCGAAGAAGAAGCAGACGCAGC</td>
</tr>
<tr>
<td>AguA_pET22R</td>
<td>GGGCTCGAGATAAATTTTCCCGCCGTGATC</td>
</tr>
</tbody>
</table>

<sup>a</sup> The two degenerate primers were designed based on highly conserved amino acid sequences (DGSIERYAG and SGKTVIQHIY) in bacterial α-glucuronidases. D represents G or A or T; H represents A or C or T; M represents A or C; N is any base; R represents G or A; W represents A or T; and Y represents T or C.

<sup>b</sup>NcoI and XhoI restriction sites are underlined.
2.2.4 Production and purification of recombinant AguA

For production and characterization of α-glucuronidase, pET22b containing the *aguA* gene was transformed into *E. coli* BL21. Transformants were selected on LB plates containing ampicillin (100 μg/mL). *E. coli* BL21 containing the *aguA* expression vector was grown at 37°C in 300 mL of LB medium supplemented with ampicillin (100 μg/mL) until the absorbance at 600 nm reached 0.6–0.8. Protein expression was carried out at 30°C with the addition of 1 mM isopropyl-β-D-thiogalactopyranoside to the culture medium. After cultivation for 3 h, *E. coli* cells were harvested by centrifugation (8,500 × g, 10 min, 4°C) and frozen at −80°C for 24 h. The frozen cell pellet was resuspended in 50 mM sodium phosphate buffer (pH 7.0), and recombinant proteins were released by sonication. Cell-free extracts were separated into lysates and cell debris by centrifugation (8,500 × g, 10 min, 4°C). The recombinant protein was purified with the Profinia Affinity Chromatography Protein Purification System using a Bio-Scale Mini Profinity IMAC cartridge and a Bio-Gel P6 desalting cartridge in accordance with the manufacturer’s instructions (Bio-Rad Laboratories, Hercules, CA, USA). Protein concentrations were determined using the Coomassie (Bradford) protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA) with bovine serum albumin as the standard. The homogeneity of the purified protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed on 5 to 20% gradient polyacrylamide gels (ATTO, Tokyo, Japan) according to the manufacturer’s instructions. Samples for SDS-PAGE were boiled for 10 min in sample buffer containing dithiothreitol (Sigma-Aldrich). After electrophoresis, gels were stained with Coomassie brilliant blue R-250 (Bio-Rad Laboratories). Molecular mass standards were from Bio-Rad Laboratories.
2.2.5 Preparation of hexenuronosyl xylotriose (ΔX3) from eucalypt kraft pulp

Hexenuronosyl xylotriose (ΔX3) was prepared from alkaline- and enzyme-treated eucalypt (*Eucalyptus globulus* L.) kraft pulp [17]. Hardwood oxygen-delignified kraft pulp (LOKP) (2 kg, obtained from Hokuetsu Kishu Paper Co., Ltd., Niigata, Japan) was soaked in 15% (w/v) NaOH (Wako Pure Chemical Industries) for 24 h at 25°C. The pulp residue was filtered and washed with water, and the filtrate was collected for further treatment as described below. After the filtrate had been neutralized with sulfuric acid, the xylan was precipitated (approximately 87 g) from the kraft pulp and separated by centrifugation (8,500 × g, 30 min, 25°C), washed with distilled water, and dried under vacuum at room temperature. The modified xylan was hydrolyzed for 72 h at 45°C and pH 4.5 (50 mM sodium acetate buffer) with xylanase (Shearzyme; Novozymes A/S, Bagsvaerd, Denmark) (544 U/g sediment) and cellulase (Onozuka R-10; Wako Pure Chemical Industries) (74 U/g sediment). Enzymatic hydrolysates were boiled for 10 min. Clear supernatants containing xylooligosaccharides were obtained by centrifugation at 8,500 × g for 30 min and applied to a packed column of activated carbon (Wako Pure Chemical Industries). The active carbon was washed with 12 L of distilled water and eluted with 9 L of 40% (v/v) ethanol. The eluted mixture was concentrated to 15 mL on a rotary evaporator. The mixture contained approximately 5.8 g of ΔX3. To avoid contamination with xylooligosaccharides such as xylotriose, the mixture containing ΔX3 was incubated with 10 units of β-xylosidase from *Bacillus pumilus* (Megazyme, Bray, Ireland) for 3 h at 35°C. The mixture was boiled for 10 min, and a clear supernatant containing ΔX3 was eventually obtained by centrifugation (16,400 × g, 15 min, 4°C). The concentration of ΔX3 was measured using high-performance anion-exchange chromatography with pulsed amperometric detection.
(HPAEC-PAD) as previously described [17]. Eventually, 5.1 g of purified ΔX3 without xylooligosaccharides was obtained from 2 kg of LOKP.

2.2.6 Enzyme activities

α-Glucuronidase activity was measured by determining the amount of glucuronic acid liberated from aldouronic acid (Megazyme) using a colorimetric assay [21]. The incubation mixture for the α-glucuronidase assay (total volume, 0.2 mL) contained 0.16 mL of substrate (2 mg of aldobiouronic, aldotriouronic, aldotetraouronic, and aldopentaouronic acids [10:60:20:10] in 0.1 M sodium acetate buffer [pH 6.0]) and 0.04 mL of the enzyme solution to be assayed. The reaction was started by addition of the enzyme. After 30 min of incubation at 40°C, the reaction was stopped by boiling the samples for 4 min. Next, 0.6 mL of copper reagent, prepared as described by Milner and Avigad [21], was added to each tube, and then the samples were boiled for 10 min and cooled on ice. Subsequently, 0.4 mL of arsenomolybdate reagent [22] was added. The samples were mixed gently, 0.8 mL of H₂O was added, and the absorbance at 620 nm was measured against H₂O. Controls were prepared by boiling a complete assay mixture at time zero, before incubation at 40°C. A substrate control was made by adding water instead of enzyme solution. A standard curve was prepared using D-glucuronic acid (Sigma-Aldrich). One α-glucuronidase unit is the amount of enzyme liberating 1 µmol of glucuronic acid per minute under standard assay conditions. The activity of α-glucuronidase towards birchwood xylan and MeGlcA (Sigma-Aldrich) was measured using the colorimetric assay after incubation at 40°C for 15 h [23]. The activity towards p-nitrophenyl-β-D-glucopyranoside (Sigma-Aldrich) was determined by measuring the absorbance of p-nitrophenol at 400 nm. The optimal pH was determined with
aldouronic acid in the following buffers at 40°C: 0.1 M sodium acetate buffer, pH 4.0 to 6.0; 0.1 M phosphate buffer, pH 6.0 to 8.0; and 0.1 M Tris-HCl buffer, pH 8.0 to 9.0. To determine pH stability, the enzyme was preincubated without substrate in buffers of different pHs for 3 h at 37°C, and then the α-glucuronidase activity was measured at 40°C for 10 min (pH 6.0). The optimal temperature was determined at pH 6.0 (50 mM sodium acetate buffer) from 30°C to 60°C. Thermostability was monitored by preincubating the enzyme without substrate in sodium acetate buffer (pH 6.0) for 3 h at 30–60°C and then assaying residual enzyme activity under standard assay conditions. Various metal ions at a concentration of 1 mM were added to the standard assay in 100 mM sodium acetate buffer (pH 6.0 at 40°C). Blanks with inactive enzyme were included to verify that the observed activity was not due to the colorimetric assay reagents. Xylanase activity was measured by determining the amount of reducing sugars released from birchwood xylan (Sigma-Aldrich) [18]. Released reducing sugars were quantified by the Somogyi–Nelson method using xylose as a standard [23]. One unit of xylanase activity was defined as the amount of enzyme that liberated 1 μmol of reducing sugar in 1 min under the assay conditions. β-Glucosidase and β-xylosidase activities were based on measurement of p-nitrophenol release from p-nitrophenyl β-D-glucoside and p-nitrophenyl β-D-xyloside (both from Sigma-Aldrich), respectively [18]. One unit of enzyme released 1 μmol equivalent of p-nitrophenol per minute.

2.2.7 Hydrolytic activity of AguA against ΔX3

Hydrolytic activity against ΔX3 was measured by directly determining the amount of xylotriose liberated from ΔX3 by HPAEC-PAD using a DX-500 series chromatograph equipped with a PAD II pulsed amperometric detector (Dionex, Sunnyvale, CA, USA).
AguA (50–60 µg/mL) was incubated with the prepared ΔX3 (4.7–6.0 mM) in 100 mM sodium acetate buffer (pH 6.0) at 40°C for 3 h, and then the reaction mixture was boiled for 10 min and cooled on ice. The xylooligosaccharides were analyzed by HPAEC-PAD with a CarboPac PA-100 column (250 mm × 4 mm) and a PA-100 guard column (25 mm × 3 mm) at 30°C. Gradient elution was performed with 100 mM NaOH and 100 mM NaOH/1 M sodium acetate at a flow rate of 1 mL/min. Authentic ΔX3, provided by Dr. Shigeki Yoshida (University of Tsukuba, Ibaraki, Japan), was used as a standard. The α-glucuronidase from *Geobacillus stearothermophilus* was purchased from Megazyme International (Bray, Ireland). D-Xylose, 1,4-β-D-xylobiose, 1,4-β-d-xylotriose, and 1,4-β-D-xylotetraose (Megazyme International) were used as external standards. The specificity of AguA for ΔX3 was determined by incubating purified enzyme (1 µg/µL) with 100 nmol of substrate in 100 µL of reaction buffer (50 mM sodium acetate, pH 6.0) at 40°C for approximately 16 h. The digested products (20 µL of a complete digest containing 20 nmol equivalents of product) were spotted on a thin-layer chromatography plate (10 by 10 cm; thickness, 0.25 mm; Silica Gel 60; Merck KGaA) along with 20 nmol of xylose or xylooligosaccharides (xylobiose, xylotriose, and xylotetraose) and HexA. The plate was developed with *n*-butanol–acetic acid–water (1:1.5:1.5, v/v) for 1.5 h, air-dried for 10 min, and sprayed with 1% (w/v) thiobarbituric acid in methanol. The stained plate was baked in an oven at 110°C for 10 min for visualization (pink color) of HexA [17].

2.2.8 Biological degradation of ΔX3 using *P. curdlanolyticus* B-6

*P. curdlanolyticus* B-6 was grown for 48 h at 37°C in 300 mL of Berg’s mineral salt medium supplemented with 0.5% (w/v) birchwood xylan as the sole carbon source. *P.*
*Curdlanolyticus* B-6 cells were harvested by centrifugation (8,500 ×g, 10 min, 4°C) and frozen at −80°C for 24 h. The culture broth was used as the extracellular fraction. The frozen cell pellet was washed with 50 mM sodium acetate buffer (pH 6.0) and resuspended in 10 mL of the same buffer. Cell-free extract was prepared by sonication and separated into lysate and cell debris by centrifugation (10,810 ×g, 10 min, 4°C). The lysate was used as the intracellular fraction. Protein concentrations were determined using the Coomassie (Bradford) protein assay kit (Thermo Fisher Scientific) with bovine serum albumin as the standard. The protein concentrations of the extracellular and intracellular fractions were estimated as 0.17 mg/mL and 2.7 mg/mL, respectively.

### 2.2.9 Nucleotide sequence accession number

The GenBank accession number for *aguA* from *P. curdlanolyticus* B-6 is KM278173.

### 2.3 Results and Discussion

#### 2.3.1 Characterization of AguA from *P. curdlanolyticus* B-6

According to the Carbohydrate-Active enZYmes (CAZy) Database, α-glucuronidases are found in three glycoside hydrolase families (GH4, GH67, and GH115). The majority of bacterial and fungal α-glucuronidases are classified within GH67. To determine whether α-glucuronidases can remove HexA side groups from xylooligosaccharides, a GH67 α-glucuronidase gene was cloned from *P. curdlanolyticus* B-6. An α-glucuronidase gene fragment (1,400 bp) was amplified by PCR using degenerate primers based on two highly conserved amino acid sequences (DGSIERGYAG and SGKTVIQHIY) (Table 1). The 5’ and 3’ flanking regions were amplified by PCR-based genome walking and assembled with the known partial sequence. The full-length
α-glucuronidase gene, aguA, contains 2,070 bp and encodes a protein of 690 amino acids with a calculated molecular mass of 77.3 kDa. The conserved amino acid sequences, DGSIERGYAG and SGKTVIQHIY, are located at amino acid residues 167–176 and 613–622, respectively. The deduced amino acid sequence encoded by aguA showed homology with putative GH67 α-glucuronidases from *Paenibacillus mucilaginosus* (69% identity, WP_014369631), *Paenibacillus* sp. oral taxon 786 (68% identity, WP_009223235), *Cohnella thermotolerans* (67% identity, WP_027092093), *Paenibacillus barengoltzii* (67% identity, WP_016312234), *Paenibacillus* sp. JDR-2 (62% identity, WP_015842945), and *Paenibacillus polymyxa* (60% identity, WP_025719411).

To characterize the enzymatic properties of AguA, recombinant AguA was expressed in *E. coli* BL21, purified to electrophoretic homogeneity using immobilized-metal affinity chromatography (Fig. 2.2), and assayed for activity with aldouronic acids and other substrates. AguA showed highly specific activity for MeGlcA side groups of aldouronic acids, while no activity was observed with birchwood xylan, 4-O-methyl-D-glucurono-D-xylan, or *p*-nitrophenyl-β-D-glucopyranoside when assayed at 40°C (pH 6.0). The optimal pH for α-glucuronidase activity of AguA was 6.0, and the enzyme was stable in the range of pH 4.0–7.0. The temperature for maximum activity was found to be 40°C at pH 6.0. These enzymatic properties and the narrow substrate specificity indicate that AguA, like other known GH67 α-glucuronidases, removes uronic acid from the nonreducing end of glucuronoxyloligosaccharides but does not attack glucuronoxylan [16, 24-26]. To measure the substrate affinity and catalytic efficiency of AguA toward aldouronic acids, kinetic parameters were determined by Michaelis–Menten analysis. Initial reaction rates
were determined from the kinetic curves of reactions containing different concentrations of aldouronic acid. The $K_m$ and $V_{\text{max}}$ values for AguA were estimated to be $1.67 \pm 0.1$ mM and $838 \pm 0.5$ μmol/(min·mg protein), respectively, corresponding to a $k_{\text{cat}}$ of 1081.8/s. The effect of various metal ions on α-glucuronidase activity was also evaluated. Although it seems unlikely that AguA requires a specific metal ion for its catalytic activity, 1 mM Co$^{2+}$, Fe$^{3+}$, Cu$^{2+}$, and Ni$^{2+}$ enhanced AguA activity by 173%, 244%, 194%, and 119%, respectively. In contrast, Mg$^{2+}$, Mn$^{2+}$, Ca$^{2+}$, Zn$^{2+}$, and K$^+$ caused losses of activity of 88%, 78%, 47%, 71%, and 70%, respectively. These metal ions are known to affect the activity of GH67 α-glucuronidases and have considerable affinity for functional groups such as thiols, imidazoles, and amines [25]. Thus, it is likely that these metal ions affect the active site and structure of AguA.
Fig. 2.2 SDS-PAGE analysis of AguA purification steps.

Recombinant protein was produced by *E. coli* harboring an expression plasmid. Lane M, standard protein molecular mass markers; lane 1, crude lysate; lane 2, flow-through; lane 3, wash; lane 4, eluate (purified protein). Lane 4 contains 3.5 μg (10 μL) of protein.
2.3.2 Removal of hexenuronic acid (HexA) groups from hexenuronosyl xylotriose (ΔX3) by AguA

To determine whether GH67 α-glucuronidase can remove HexA from hexenuronosyl xylooligosaccharides, hexenuronosyl xylotriose (ΔX3) was prepared from eucalyptus kraft pulp and used as a model substrate. The fractionation was carried out as described by Winyasuk et al. [17] (Fig. 2.3). After treatment with commercial xylanases and cellulases, HPAEC-PAD analysis showed that the fraction of ΔX3 containing xylooligosaccharides still contained xylotriose (Fig. 2.4). To clearly observe the release of xylotriose from ΔX3 by AguA, the ΔX3 fraction containing xylotriose was treated with commercial β-xylosidase. Free xylotriose in this fraction was converted to xylose, but the ΔX3 concentration was not affected (Fig 3b), indicating that β-xylosidase cannot degrade xylotriose decorated with HexA.

HPAEC-PAD analysis showed that xylotriose was liberated when AguA was incubated with ΔX3, and the ΔX3 peak decreased with the appearance of xylotriose (Fig. 2.5). To confirm whether the peak observed following incubation with AguA was xylotriose, β-xylosidase was added to the reaction (Fig. 2.5). The xylotriose was completely converted to xylose by the β-xylosidase. These results clearly indicated that xylotriose was liberated from ΔX3 by AguA, which can hydrolyze the α-1,2 glycosidic
bond even when the side group is HexA instead of MeGlcA (Fig. 2.5). The $K_m$ for $\Delta X3$ was estimated to be $4.6 \pm 0.5$ mM. The affinity of AguA for $\Delta X3$ was only slightly lower than that for MeGlcA-substituted aldouronic acids. However, $V_{\text{max}}$ for the release of xylotriose from $\Delta X3$ was $5.0 \pm 0.6 \mu\text{mol xylotriose/(min·mg protein)}$, and the $k_{\text{cat}}$ was estimated to be $6.4$/s. These results indicate that although the affinity of AguA for $\Delta X3$ was not different from that for MeGlcA-substituted aldouronic acids, the velocity and efficiency of AguA were significantly affected by HexA side groups. Recently, the three-dimensional structure and biochemical properties of a GH67 $\alpha$-glucuronidase (GlcA67A) from Pseudomonas cellulosa (Cellvibrio japonicus) were reported [15]. GlcA67A (GenBank accession No. CP000934) has an amino acid similarity of 46% with AguA. The bacterial $\alpha$-glucuronidases belonging to GH67 catalyze bond cleavage by an SN2-like single displacement mechanism in which water attacks the anomeric carbon of the uronic acid, concomitant with protonation of the glycosidic oxygen and departure of the leaving group, leading to an inversion of the aromatic configuration and generation of the $\beta$-anomer of MeGlcA [16, 27]. Co-crystallization of GlcA67A with glucuronic acid revealed a binding site within a deep, partially hydrophobic pocket in the enzyme surface [15]. The hydrophobic pocket provides exquisite recognition elements for the carboxylate moiety of glucuronic acid and may accommodate a
4-\(O\)-methyl substituent through surrounding hydrophobic amino acid residues such as Val210 and Trp160 [15]. Thus, if the 4-\(O\)-methyl substituent is lost by \(\beta\)-elimination of methanol during alkaline pulping, the binding of the substrate in the hydrophobic pocket might be unstable.

Thin-layer chromatography (TLC) was also carried out to confirm the release of HexA in reactions with or without AguA. Deoxy sugars such as HexA yield a red spot on TLC plates developed with thiobarbituric acid, but no color is observed for \(\Delta X3\) [17, 28]. Although the reaction of \(\Delta X3\) with boiled AguA did not show a red spot on a TLC plate sprayed with thiobarbituric acid, the reaction of \(\Delta X3\) with native AguA did show a red spot (Fig. 2.6). These results strongly support the detection of xylotriose in the reaction with AguA by HPAEC-PAD analysis. To determine whether other bacterial \(\alpha\)-glucuronidases belonging to GH67 can release HexA from \(\Delta X3\), we performed the hydrolysis tests using the well-characterized GH67 \(\alpha\)-glucuronidase from \textit{G. stearothermophilus} [25, 29, 30]. Because this \(\alpha\)-glucuronidase is commercially available as a high-purity recombinant enzyme from Megazyme International, it might give more reproducible results than non-commercial preparations. Complete three-dimensional structures are available for only two GH67 enzymes [30]. GlcA67A from \textit{C. japonicus} and the \(\alpha\)-glucuronidase from \textit{G. stearothermophilus} [29]. The overall structures of
these two enzymes appear to be quite similar [30]. The α-glucuronidase from *G. stearothermophilus* also hydrolyzed ΔX3 to xylotriose and HexA, but it had a lower $V_{\text{max}}$ (2.7 µmol xylotriose/(min·mg protein)) than AguA. These results suggest that GH67 α-glucuronidases may be useful for the specific removal of HexA in kraft pulping processes.
Fig. 2.3 Scheme for the fractionation of $\Delta X_3$

A total of 5.1 g of purified $\Delta X_3$ was obtained from 2 kg of hardwood oxygen-delignified kraft pulp (LOKP).

<table>
<thead>
<tr>
<th>HexA content (mmol/kg) (Yoon, K., 2011)</th>
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</thead>
<tbody>
<tr>
<td>Untreated LOKP</td>
</tr>
<tr>
<td>43.9</td>
</tr>
</tbody>
</table>
Fig. 2.4 ΔX3 treatment with β-xylosidase

HPAEC-PAD chromatograms for the fractionation of ΔX3 before (top) and after (bottom) treatment with β-xylosidase. The PAD response (nC) and elution time (min) are shown on the y- and x-axis, respectively. The dotted lines in the chromatograms show the concentration of eluent.
Fig. 2.5 HPAEC-PAD analyses of xylotriose

Three HPAEC-PAD chromatograms show analysis of reaction mixtures of ΔX3 without AguA (top), with AguA (middle), and following addition of β-xylosidase after hydrolysis with AguA (bottom). The PAD response (nC) and elution time (min) are shown on the y- and x-axis, respectively. The dotted lines in the chromatograms show the concentration of eluent.
Fig. 2.6 TLC of HexA released from ΔX3 by AguA.

HexA was detected on TLC plates sprayed with thiobarbituric acid solution.
2.3.3 Possibility of biological degradation using *P. curdlanolyticus* B-6

An attempt to directly remove HexA from ΔX3 using intracellular enzymes from *Paenibacillus* sp. strain 07-G-dH has been reported [17]. The absence of an N-terminal signal sequence indicates that AguA of *P. curdlanolyticus* B-6 might be an intracellular enzyme. The majority of α-glucuronidases belonging to GH67 have been reported to be either membrane-bound or intracellular enzymes [16, 24-26, 31]. To characterize the enzymatic properties of *P. curdlanolyticus* B-6 in relation to ΔX3 degradation, extracellular and intracellular fractions were prepared from cells cultured with birchwood xylan as the sole carbon source. α-Glucuronidase and β-xylosidase activities in the intracellular fraction extracted from *P. curdlanolyticus* B-6 cells were considerably higher than those in the culture supernatant (Table 2.2). These results indicate that the GH67 α-glucuronidase of *P. curdlanolyticus* B-6 may be an intracellular enzyme [18]. To explore the possibility of direct biological bleaching using α-glucuronidase, the ability of the intracellular fraction to degrade ΔX3 was assessed. Although only a small amount of xylotriose was released when the intracellular fraction was incubated with ΔX3, an increase in the amount of xylose occurred in connection with a decrease in the amount of ΔX3 (Fig. 2.7). In contrast, when the extracellular fraction (culture broth) was incubated with ΔX3, no increase in xylose or xylotriose
concentration or decrease in ΔX3 concentration was observed (data not shown). These results may be understood as the complete degradation of ΔX3 by the synergistic activity of intracellular α-glucuronidase and β-xylosidase. The reaction catalyzed by AguA—the removal of HexA from ΔX3—may be the rate-limiting step in hydrolysis of ΔX3. The released xylotriose should be quickly converted to xylose monomers by β-xylosidase. The pattern of these end products is similar to that observed when intracellular enzymes from Paenibacillus sp. strain 07-G-dH [17] were tested for degradation of ΔX3.
Table 2.2 Enzymatic properties of extracellular and intracellular fractions from *P. curdlanolyticus* B-6

<table>
<thead>
<tr>
<th>Enzymatic activity</th>
<th>Extracellular fraction(^a)</th>
<th>Intracellular fraction</th>
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<tr>
<td>(\alpha)-Glucuronidase(^b)</td>
<td>0.0027±0.0002</td>
<td>0.038±0.001</td>
</tr>
<tr>
<td>(\beta)-Xylosidase</td>
<td>0.0031±0.003</td>
<td>0.040±0.005</td>
</tr>
<tr>
<td>(\beta)-Glucosidase</td>
<td>0.0014±0.0002</td>
<td>0.007±0.0009</td>
</tr>
<tr>
<td>Xylanase</td>
<td>5.51±1.5</td>
<td>0.73±0.08</td>
</tr>
</tbody>
</table>

Values are means of triplicate experiments ± standard deviation.

\(^a\)Culture broth from a xylan-grown culture was used directly as the enzyme preparation for each assay.

\(^b\)Aldouronic acid was used as substrate.
Biological degradation of $\Delta X_3$ was carried out using intracellular extract from xylan-grown *P. curdlanolyticus* B-6 cells. Concentrations of $\Delta X_3$ (triangles), xylotriose (diamonds), and xylose (squares) were measured by HPAEC-PAD analysis. The concentration of released HexA (circles) was estimated from the decrease in the concentration of $\Delta X_3$. 

Fig. 2.7 Biological degradation of $\Delta X_3$ using extract from *P. curdlanolyticus* B-6.
2.4 Conclusions

The presence of HexA negatively affects pulps by decreasing brightness. To determine whether HexA-decorated xylooligosaccharides could be hydrolyzed by α-glucuronidase, AguA, a GH67 α-glucuronidase from P. curdlanolyticus B-6, was characterized for its ability to degrade ΔX3 as a model substrate. When ΔX3 was incubated with AguA, xylotriose and HexA were released and accumulated in the reaction mixture. ΔX3 can be directly degraded by the α-glucuronidase activity of intracellular extract from P. curdlanolyticus B-6. These findings provide new insight into the direct biological degradation of ΔX3 by α-glucuronidase and may help reduce the consumption of active bleaching chemicals. This is the first report of direct degradation of ΔX3 by a GH67 α-glucuronidase.

2.5 References


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Chapter 3

Characterization of hexuronosyl xylan-degrading enzymes produced by *Paenibacillus* sp.

Overview

*Paenibacillus* sp. strain 07 was used to identify the enzyme involved in hexenuronic acid (HexA) removal from kraft pulp. The extracellular and intracellular enzymes of *Paenibacillus* sp. were assessed for hexuronosyl-xylotriose (ΔX3) degradation activity. First, ΔX3 was obtained from hardwood kraft pulp by enzymatic hydrolysis using three commercial enzymes. Crude extracellular and intracellular enzyme fractions were obtained from *Paenibacillus* cultures cultivated using 0.5% birch-wood xylan (w/v) as the sole carbon source. The enzyme fractions were used to assess ΔX3-degrading activity by performing hydrolysis assays. Crude enzyme activity was performed using ΔX3 as the substrate in sodium acetate buffer, pH 6, at 50°C. Reaction products were analyzed by HPAEC-PAD. The assay data showed that the enzyme fractions had different chromatogram patterns. Chromatogram data indicated that xylose and hexuronosyl xylobiose (ΔX2) peaks were generated after treatment with the intracellular enzyme fraction. The chromatogram patterns of the extracellular fraction assays indicated that xylose, xylotriose, and ΔX2 were produced. Thus, the intracellular enzymes of *Paenibacillus* can hydrolyze the xylosidic linkages at the reducing ends of ΔX3, whereas the extracellular enzyme fraction has a specific enzyme that can hydrolyze HexA, which can be further used for HexA removal during bio-bleaching.
3.1 Introduction

Hemicellulose is re-evaluated to be an important wood component that influences pulp yields, strength properties and pulp-bleaching ability [1]. During the heating treatment step in kraft pulping of wood chips, about 50% of 4-O-methylglucuronic acid group residues in xylan are converted to hexenuronic acid (HexA) groups by β-elimination reactions [2,3]. The HexA groups inflate the measurement of pulp kappa number (KN), which indicates the lignin content of the pulp and is useful for estimating the quantity of bleaching agents required to treat the pulp. HexA inflates the KN by approximately 3–6 KN units for hardwood pulps and by 1–3 KN units for softwood pulps [2-5]. Thus, the presence of HexA in unbleached pulp has adverse effects on pulp bleaching operations, as it increases the amounts of bleaching chemicals required and decreases the brightness stability of the pulp [6,7].

Previous efforts have been focused on the removal of HexA from kraft pulp [8,9]. Chlorine dioxide bleaching is commonly used for bleaching oxygen-bleached kraft pulp. Chlorine dioxide is not very efficient in degrading HexA. However, the chlorine (Cl₂) formed during this stage may chlorinate HexA and other structure of lignin and carbohydrates. The chlorinated form of HexA prevents the post yellowing reactions of HexA, and these chloro-organic compounds may have negative environmental effects [9]. The problems associated with HexA removal have led to the development of a hot chlorine dioxide bleaching stage or a hot acid treatment stage, which leads to lowered cellulose viscosity [9]. Thus, a HexA-removal method that is highly specific and uses mild treatment conditions is required.

Enzymatic degradation of HexA has been considered for developing methods for HexA removal [10,11]. Enzyme-based methods have several advantages over
chemical-based methods. For example, enzymes have high specificity and are, therefore, less likely to act on unwanted by-products and yield better pulp quality. In addition, enzymatic reactions do not require high reaction temperatures and are energy saving and ecofriendly [11]. Thus, enzyme-based methods can be potentially used for implementing clean bleaching processes and for developing high-added value products.

Selective HexA removal from pulp by using HexA-degrading enzymes can yield better quality pulp. Winyasuk et al (2012) showed that a soil bacterium, *Paenibacillus* sp. strain 07-G-dH (*Paenibacillus* sp. strain 07), has the ability to utilize HexA-substituted xylotriose (hexenuronosyl xylotriose, ΔX3) [12]. In this study, ΔX3, a key model compound of hexenuronoxylan, was used to determine whether the enzymes secreted by a *Paenibacillus* strain could hydrolyze HexA in pulp. In addition, we characterized the extracellular and intracellular enzymes secreted by *Paenibacillus* strain.

### 3.2 Materials and methods

#### 3.2.1 Bacterial strain and medium

*Paenibacillus* sp. strain 07-G-dH (hereafter referred to as *Paenibacillus* sp. strain 07) was provided by Dr. Shigeki Yoshida (University of Tsukuba, Ibaraki, Japan). *Paenibacillus* sp. strain 07 was grown on a production medium (pH 7.0) described by Winyasuk et al (2012) [18, 20]. Briefly, the medium contained 1 g of yeast extract, 1 g of polypeptone, 1 g of yeast nitrogen base, 1 g of KH$_2$PO$_4$, and 1 g of MgSO$_4$.7H$_2$O and was supplemented with 5 g of birch-wood xylan (Sigma-Aldrich, St. Louis, MO, USA) per liter of distilled water (pH 7.0). Chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan), Nihon Seiyaku and Difco BD Laboratories.
3.2.2 Preparation of ΔX3 by enzymatic hydrolysate of kraft pulp xylan

*Eucalyptus* oxygen-bleached kraft pulp (obtained from Hokuetsu Kishu Paper Co., Ltd., Niigata Mill, Japan) was soaked in 15% (w/v) NaOH for 24 h, and then filtered using cotton cloth. The filtrate was neutralized using sulfuric acid (H₂SO₄), and the pH of the filtrate was adjusted to pH 7. The supernatant was separated from the pellet by centrifugation at 8500 × g for 30 min. The pellet containing modified xylan was suspended in distilled water and then dried under vacuum at room temperature. Modified xylan was hydrolyzed using xylanase (Shearzyme; Novozymes A/S, Bagsvaerd, Denmark) (544 U/g) and “Onozuka” R-10 from *Trichoderma viride* (Yakult Pharmaceutical Industry Co., LTD, Japan) (74 U/g) at pH 4.5 (50 mM acetic acid buffer) and 45°C for 72 h. The mixture was heated at 100°C for 10 min and centrifuged. The supernatant was applied to a column packed with activated carbon (Wako Pure Chemical Industries) for chromatography, at a flow rate of 60 mL/h. Next, the column was washed with 12 L of distilled water to remove the monomers. After all monomers were removed from the column, oligosaccharides were eluted further with 40% ethanol aqueous solution. The eluate was concentrated using a rotary vacuum evaporator. To eliminate contamination with xylooligosaccharides (XOs), ΔX3 mixture was purified further by treating the eluate with β-xylosidase (10 U/mL) obtained from *Bacillus pumilus* (Megazyme, Bray, Ireland); the enzyme treatment was performed at pH 7.0 (100 mM phosphate buffer), 35°C for 3 h. The purity and concentration of ΔX3 were analyzed using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD).
3.2.3 Preparation of extracellular and intracellular enzymes from *Paenibacillus* sp. strain 07

*Paenibacillus* cells were grown on production medium supplemented with 0.5% (w/v) birch-wood xylan (Sigma-Aldrich) as the sole carbon source for 1 day at 37°C. The culture supernatant was separated from the bacterial cells by centrifugation (7800 × g, 15 min, 4°C) and precipitated using 80% ammonium sulfate for 24 h at 4°C. The pellet obtained from this step was harvested by centrifugation (7800 × g, 45 min, 4°C), suspended in MilliQ water, and dialyzed using Econo-Pac® 10 DG Desalting column (Bio-Rad) against the same solution at 4°C. This suspension was used as the extracellular fraction.

The cell pellets were frozen at −80°C for 24 h. Frozen pellets were thawed, washed using phosphate buffer saline (pH 7), and resuspended in 30 mL of the same buffer. Cell-free extract was prepared by sonication for 30 min. The sonicated cells were separated into cell debris and lysate by centrifugation. The lysate was used as the intracellular fraction.

3.2.4 Hydrolytic activity of the enzyme fraction against ΔX3 and enzymatic product analysis

Hydrolysis assay for ΔX3 by the using extracellular and intracellular fractions was performed in 50 mM sodium acetate buffer (pH 6) at 50°C for 6 h, and then stopped by heating the reaction mixture at 100°C for 10 min. Enzyme fractions (30-90 µg/mL) was incubated with the prepared ΔX3 (3.5 mM), and then the extracellular and intracellular hydrolysis products were analyzed by HPAEC-PAD DX-500 series chromatograph with a PAD II pulsed amperometric detector (Dionex, Sunnyvale, CA, USA). This machine
equipped with a CarboPac® PA100 analytical column (250 mm × 4 mm). The column was equilibrated with 100 mM NaOH at 30°C with a flow rate of 1 mL/min. Gradient elution was performed using 100 mM NaOH and 100 mM NaOH/1 M CH₃COONa. Authentic ΔX3, provided by Dr. Shigeki Yoshida (University of Tsukuba, Ibaraki, Japan) was used as a standard.

3.2.5 Enzyme activities

α-Glucuronidase activity from the extracellular and intracellular fractions of *Paenibacillus* sp. strain 07 was measured by determining the amount of glucuronic acid liberated from aldouronic acid (Megazyme) using a colorimetric assay [13]. The incubation mixture for the α-glucuronidase assay (total volume, 0.2 mL) contained 0.16 mL of substrate (2 mg in 100 mM sodium acetate buffer, pH 6.0) and 0.04 mL of the enzyme solution to be assayed. The reaction was started by adding the enzyme. After 30 min of incubation at 40°C, the reaction was stopped by boiling the samples for 4 min. Next, 0.6 mL of copper reagent, prepared as described by Milner and Avigad (1967), was added to each tube, and then, the samples were boiled for 10 min and cooled on ice. Subsequently, 0.4 mL of arsenomolybdate reagent was added [14]. The samples were mixed gently, 0.8 mL of H₂O was added, and the absorbance at 620 nm was measured against H₂O. Controls were prepared by boiling a complete assay mixture at time 0, before incubation at 40°C. A substrate control was made by adding water instead of enzyme solution. A standard curve was prepared using D-glucuronic acid (Sigma-Aldrich). One α-glucuronidase unit is the amount of enzyme liberating 1 μmol of glucuronic acid per minute under standard assay conditions. The activity of the extracellular and intracellular fractions toward xylan was measured using the colorimetric assay [23]. The activity toward 4-nitrophenyl β-D-xylopyranoside
and 4-nitrophenyl β-D-glucopyranoside (Sigma-Aldrich) was determined by measuring the absorbance of 4-nitrophenol at 410 nm.

3.3 Results and discussion

3.3.1 Preparation of ΔX3 as a model compound of HexA

ΔX3 was prepared from eucalyptus kraft pulp; fractionation was carried out as described by Winyasuk [12]. ΔX3 used in this study was produced in a two-stage sequence: (1) alkaline extraction followed by (2) enzymatic hydrolysis. In the alkaline extraction process, 87 g of xylan was obtained from 2 kg of kraft pulp. Xylan was derived from alkali-extracted precipitate during the neutralization process, using acid solution.

In the enzymatic hydrolysis process, two commercial enzymes were used (Shearzyme and Cellulose R10). Since xylan is a heteropolymer with a homopolymeric backbone composed of β-1,4-linked xylose units and various branching units, the synergistic action of different enzymes is needed to completely hydrolyze the complex xylan structures. In this study, xylan was first hydrolyzed with xylanase “Shearzyme,” which mainly contains endo-xylanase. The endo-β-1, 4-xylanases (primarily from GH10) degrade xylan by attacking the β-1,4-bonds between xylose units to produce XOs [15]. GH10 xylanases liberate shorter XOs products than those released by other xylanase (GH11). In contrast, GH11 produces longer XOs because the activity of this enzyme is abrogated by additional groups, which restrict the access to β-1,4-linkages in the xylan backbone [15,16]. In addition, GH10 xylanases can act near the substituted xylose residue forming XOs that carry the substituent at the non-reducing terminal xylopyranose residue. Consequently, GH10 treatment liberates ΔX3 as the shortest
acidic oligosaccharides. The hydrolysate from this step contains xylose, XOs, and several kinds of acidic oligosaccharides. These mixtures can then be hydrolyzed further with cellulase “Onozuka” R10 that contains β-xylosidase and α-glucuronidase [3,12,17]. β-Xylosidase converts XOs with lower degree of polymerization (DP) into monomeric xylose. In addition, debranching enzymes such as α-glucuronidase are needed to cleave xylan side groups (4-O-methyl-D-glucuronic acid).

Hydrolysate from the previous step was purified further and separated from undesirable compounds by using charcoal column chromatography followed by elution with ethanol. The charcoal was used to separate monosaccharides and disaccharides from XOs. Charcoal chromatography was used as the preferred method for sugar purification because of its high loading capacity [18]. The XOs remaining in the column were then eluted using 40% ethanol in water to fractionate XOs based on their molecular weight. A hydrolysate containing 5.8 g of ΔX3 was obtained. The structure of ΔX3 was characterized in a previous study [3, 12].

After treatment with commercial xylanases and cellulases, HPAEC-PAD showed that the ΔX3 fraction contained xylotriose (Fig. 3.1). The remnant ΔX3 were expected because of the difficulty associated with separation of XOs from high-DP XOs and from acidic oligosaccharides (XOs with uronic acid substituents). Next, contaminated ΔX3 were purified further using β-xylosidase. After hydrolysis, ΔX3 were analyzed, and the data showed that the xylotrioses were successfully removed from the substrate without losing ΔX3. These data were in agreement with those of previous studies conducted by Tenkanen et al [19] and Biely et al [16]. Liberated glucuronoxylan was hydrolyzed by xylanase GH10 and was resistant to β-xylosidase. These findings indicate that the reducing end of liberated glucuronoxylan contains a xylopyranosyl residue substituted with MeGlcA. β-Xylosidases are enzymes that successively remove
the terminal xylose unit from the non-reducing end of XOs [19]. In addition, removal of xylotriose from the ΔX3 fraction is needed because the target enzyme mainly produced xylotriose by cleaving the 1,2-linkage of ΔX3. The chromatograms of β-xylosidase-treated ΔX3 are shown in Fig. 3.1. This step yielded 5.1 g of ΔX3.
Fig. 3.1 HPAEC chromatograms of hexenuronosyl-xylotriose (ΔX3) before (top) and after (bottom) treated by β-xylosidase

Legend: The PAD response (nC) and retention time (min) are shown on the Y-axis and X-axis, respectively.
3.3.2 Characterization of intracellular and extracellular enzymes from *Paenibacillus* sp. strain 07

The activities of the extracellular and intracellular enzymes of *Paenibacillus* sp. strain 07 were examined using ΔX3 as the substrate. ΔX3 was hydrolyzed by the two fractions obtained from cell cultures by using birch-wood xylan as a sole of carbon source. The intracellular fraction of *Paenibacillus* sp. strain 07 rapidly degraded the substrate and afforded two major products, xylose and ΔX2, after 30 min of hydrolysis (Fig. 3.2). The ΔX3 degradation rate of the intracellular fraction was higher than that of the extracellular fraction. The substrate was completely degraded by the intracellular fraction within 2 h of hydrolysis. The HPAEC-PAD data indicated that the ΔX2 levels (indicated by peaks in the chromatogram) increased with longer hydrolysis periods, and that the ΔX3 peaks disappeared after 3 h of hydrolysis (Fig. 3.3). This finding is in agreement with those of a previous study conducted by Winyasuk et al [12]. The intracellular enzyme fraction contained reducing end xylose-releasing exo-oligoxylanase that degrades the first xylosidic linkage from the reducing-end site of ΔX3.

Likewise, glycoside hydrolases belonging to family 8 [20] and family 5/30 [21] were also known to hydrolyze xylooligosaccharides, releasing xylose from their reducing ends, but do not have activities of endo-β-1,4-xylanase and any other polymeric substrates such as chitosan, lichenan, and carboxymethylcellulose as unique properties [20, 21]. These enzymes are also involved intracellular xylan metabolism by cleaving xylooligosaccharides [20, 21]. Thus, *Paenibacillus* sp. strain 07 may possess a similar glycoside hydrolase related to the intracellular xylan metabolism, and play a role as a key enzyme for degradation of ΔX3.

The crude extracellular fraction was incubated with ΔX3 and the reaction
yielded different product patterns (Figs. 3.4 and 3.5). Chromatogram data showed that hydrolysis products contain one major product (xylose) and the other two products (xylotriose and ΔX2; Fig. 3.5). Xylose liberation in the hydrolysis reaction was increased in association with hydrolysis time, whereas an increase of ΔX2 concentration could not be observed with a significant difference even though xylose would be liberated from ΔX3. On the other hand, xylotriose concentration increased during the first hour of hydrolysis reaction and then decreased (Fig. 3.4), indicating that the released xylotriose should be quickly converted to xylose monomers by the extracellular β-xylosidase.

These phenomenon suggests that the extracellular fraction should contain mainly two enzymes as follows: a) a new enzyme that specially can degrade HexA from ΔX3 and release xylotriose; and b) a β-xylosidase that can hydrolyze xylotriose to yield xylose. We recently confirmed an α-glucuronidase from P. curdlanolyticus B-6 can remove the HexA side group from ΔX3. Interestingly, α-glucuronidase activity could not be detected in the extracellular fraction of Paenibacillus sp. strain 07 (Table 3.1), suggesting that the strain 07 may be able to produce a new enzyme.

The new HexA-liberating enzyme may have important applications because this enzyme cleaves α-1, 2-linkages between the xylose unit of the xylan chain and carboxylic acid side groups (HexA). The activity test data indicated that the new enzyme was distinct from α-glucuronidase (Table 3.1). The ΔX3 degrading enzyme system of Paenibacillus sp. strain 07 is summarized in Fig. 3.6. The enzyme activity profiles of the intracellular and extracellular fractions of Paenibacillus sp. strain 07 indicate that the fractions contain two or more unidentified enzymes, a reducing end xylose-releasing exo-oligoxylanase such as enzymes belonging to (GH8 or GH5/30) and a HexA-liberating enzyme. The uncharacterized enzymes have enzyme activities
that may be useful in the pulp bleaching process.
Fig. 3.2 Biological degradation of ΔX3 using the intracellular enzyme of *Paenibacillus* sp. strain 07

Concentrations of ΔX3 (triangles), ΔX2 (circles), xylose (diamonds), and xylotriose (squares) were measured by HPAEC-PAD analysis.
Fig. 3.3 HPAEC chromatograms of ΔX3 before (top) and after (bottom) hydrolysis using intracellular fraction of *Paenibacillus* sp. strain 07

Legend: The PAD response (nC) and retention time (min) are shown on the Y-axis and X-axis, respectively.
Fig. 3.4 Biological degradation of ΔX3 using extracellular enzyme of *Paenibacillus* sp. strain 07

Concentrations of ΔX3 (triangles), xylose (diamonds), and xylotriose (squares) were measured by HPAEC-PAD analysis.
Fig. 3.5 HPAEC chromatograms of hexuronosyl-xylotriose (ΔX3) before (top) and after (bottom) hydrolyzed by extracellular fraction of *Paenibacillus* sp. strain 07

Legend: The PAD response (nC) and retention time (min) are shown on the Y-axis and X-axis, respectively.
Fig. 3.6 ΔX3-degrading enzymes in the extracellular and intracellular enzyme fractions of *Paenibacillus* sp. strain 07
Table 3.1 Enzymatic properties of extracellular and intracellular fractions from *Paenibacillus* sp. strain 07

<table>
<thead>
<tr>
<th>Enzymatic activity</th>
<th>Specific activity (U/mg protein)</th>
<th>Extracellular fraction</th>
<th>Intracellular fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Glucuronidase&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
<td>0.023 ± 0.001</td>
<td></td>
</tr>
<tr>
<td>β-Xylosidase&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.005 ± 0.003</td>
<td>0.034 ± 0.005</td>
<td></td>
</tr>
<tr>
<td>β-Glucosidase&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.005 ± 0.001</td>
<td>0.034 ± 0.002</td>
<td></td>
</tr>
<tr>
<td>Xylanase</td>
<td>1.79 ± 0.14</td>
<td>0.30 ± 0.01</td>
<td></td>
</tr>
</tbody>
</table>

Values are means of triplicate experiments ± standard deviation.

<sup>a</sup> Aldouronic acid was used as substrate.

<sup>b</sup> 4-nitrophenyl β-D-xylopyranoside was used as substrate.

<sup>c</sup> 4-nitrophenyl-β-D-glucopyranoside was used as substrate.
3.4 Conclusions

Our data indicate that the crude intracellular enzyme fraction obtained from *Paenibacillus* sp. strain 07 contained an enzyme that released xylose residues from the reducing ends of ΔX3. The crude extracellular enzyme fraction obtained from *Paenibacillus* sp. strain 07 contained a HexA-liberating enzyme, indicated by the production of xylotriose. The released xylotriose was quickly converted into xylose monomers by the extracellular β-xylosidase.

3.5 References


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Chapter 4

General Conclusion

HexA has significant role in the bleaching process by decreasing quality of bleached pulp. Some attempts have been conducted to reduce the HexA content in the pulp using chemical and biological treatments. These technologies still have the limitations related with the environmental problems, pulp quality and specificity of the enzyme. The need of enzyme with high selectivity to remove HexA from kraft pulp is a critical problem. Hence, there is an immediate need to examine enzymes for more efficient and direct enzymatic removal of HexA from kraft pulp. In this study, HexA degrading enzyme from *Paenibacillus* species have been conducted using ΔX3 as a substrate. Based on the result there was two enzyme systems for HexA removal.

First of all, a GH67 α-glucuronidase (AguA) was found as a key enzyme for HexA removal in *Paenibacillus curdlanolyticus* B-6. HexA removal can be achieved by synergistic action between intracellular AguA and β-xylosidase. When ΔX3 was incubated with AguA, xylotriose and HexA were released and accumulated in the reaction mixture. The released xylotriose then quickly converted to xylose monomers.
by β-xylosidase. These results showed that α-glucuronidase GH67 was able to remove HexA from ΔX3. These findings provide new insight into the direct biological degradation of ΔX3 by α-glucuronidase and may help reduce the consumption of active bleaching chemicals. This is the first report of direct degradation of ΔX3 by a GH67 α-glucuronidase.

Meanwhile another HexA degradation mechanism was found when ΔX3 was incubated with extracellular fraction of *Paenibacillus* sp. strain 07. Based on the chromatogram result there was xylose, xylotriose and ΔX2 liberation in the hydrolysis product. This phenomenon suggests that degradation mechanism of HexA can be achieved using synergistic enzyme between at least two enzymes, an enzyme that specially can degrade HexA from ΔX3 and release xylotriose and β-xylosidase that can hydrolyze xylotriose to yield xylose. This enzyme might be different from α-glucuronidase, because activity of this enzyme could not be detected in the extracellular fraction of *Paenibacillus* sp. strain 07.

The finding of HexA degrading enzyme in the intracellular and extracellular fraction of *Paenibacillus* species marked the beginning of the development enzyme systems for pulp bleaching technology. Application of these enzymes as pre bleaching agents or as a part in the bleaching sequences may be a promising technology for direct
removal of HexA from kraft pulp compare with other enzyme application. When these specific HexA degrading enzymes applied for direct removal of HexA during kraft pulp bleaching, several advantages will obtained regarding to improvement of pulp quality, save of bleaching chemicals and better performances of pulp and paper industry related with environmental concern due to decreasing of toxic compounds formation. To take advantages of these prospects, several future studies will needed such as improvement of HexA degrading enzyme production, further characterization of two or more unidentified enzymes in *Paenibacillus* sp. strain 07 that may be useful for bleaching process. The goals are direct application of this HexA degrading enzyme in the bleaching process.
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I would like to dedicate my dissertation to my Father, thank you for your believe that I can be a Doctor someday, and my Grandfather that always said I know you can do anything, just try your best to get i
APPENDIX A

Fig. A Construction, production and purification of recombinant AguA from *Paenibacillus curdlanolyticus* B-6
APPENDIX B

Table 2. Comparison of kinetics data between aldouronic acids and hexenuronosyl xylotriose

<table>
<thead>
<tr>
<th>Substrates</th>
<th>$K_m^1$ (mM)</th>
<th>$V_{max}^2$ (µmol/min.mg protein)</th>
<th>$K_{cat}^3$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aldouronic acids</td>
<td>1.67</td>
<td>838</td>
<td>1081.8</td>
</tr>
<tr>
<td>Hexenuronosyl-xylotriose</td>
<td>4.6</td>
<td>5.0</td>
<td>6.4</td>
</tr>
</tbody>
</table>

1) $K_m$ (Michaelis constant): to measure affinity of the enzyme to the substrate

High $K_m$: enzyme has a low affinity for its substrate, and requires a greater concentration of substrate to achieve $V_{max}$

2) $V_{max}$: reflects how fast the enzyme can catalyze the reaction

3) $K_{cat}$: the "turnover number", maximum number of molecules of substrate that an enzyme can convert to product per catalytic site per unit of time
APPENDIX C

Fig. B Liberation of xylotriose from hexenuronosyl xylotriose (ΔX3) by recombinant
α-glucuronidase of P. curdlanolyticus B-6.

Hydrolysis was carried out at pH 6.0 and 40 °C.