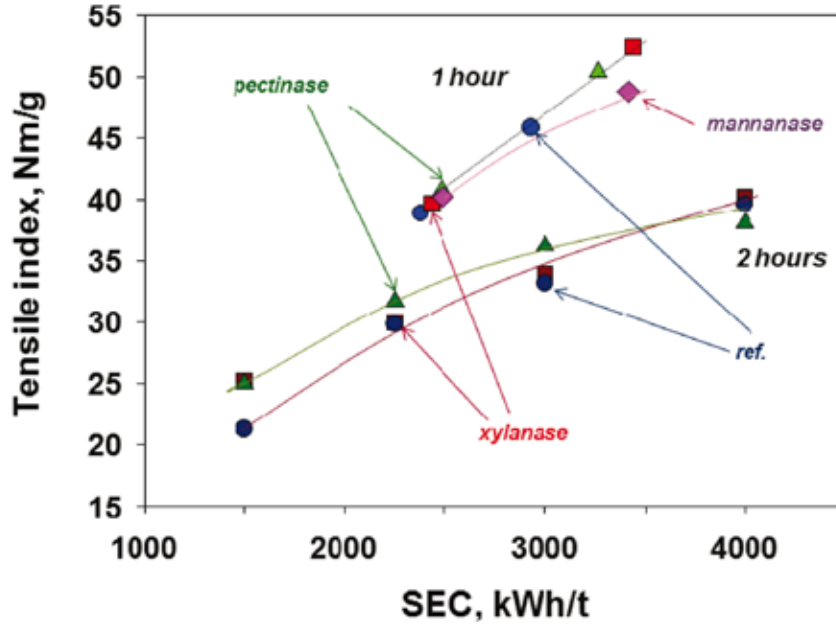
CRUW Mechanical Pulping

Sub-project 10: Enzyme treatment of chips for energy reduction in TMP

Lennart Salmén, *Innventia;* **Paul Ander, Dinesh Fernando, Geoffrey Daniel,** *SLU;* **Silvia Viforr,** *Innventia;* **Tomas Mårtensson,** *Innventia*

Lars Hildén, *Holmen;* **Anders Moberg,** *Stora Enso;* **Magnus Paulson,** *Akzo Nobel;* **Erik Nelsson,** *Holmen;* **Roland Bäck,** *SCA;* **Peter Sandström,** *SCA*



Intern rapport nr 10 (begränsad spridning)

CRUW
Centre for Research on Ultrastructure of Wood fibres
Centrum för forskning om Vedfiberns Ultrastruktur
Sveriges lantbruksuniversitet
Institutionen för skogens produkter
Uppsala 2013

CRUW Mechanical Pulping

Sub-project 10: Enzyme treatment of chips for energy reduction in TMP

Lennart Salmén, *Innventia;* **Paul Ander, Dinesh Fernando, Geoffrey Daniel,** *SLU;* **Silvia Viforr,** *Innventia;* **Tomas Mårtensson,** *Innventia*

Lars Hildén, *Holmen;* **Anders Moberg,** *Stora Enso;* **Magnus Paulson,** *Akzo Nobel;* **Erik Nelsson,** *Holmen;* **Roland Bäck,** *SCA;* **Peter Sandström,** *SCA*

Intern rapport nr 10 (begränsad spridning)

CRUW
Centre for Research on Ultrastructure of Wood fibres
Centrum för forskning om Vedfiberns Ultrastruktur
Sveriges lantbruksuniversitet
Institutionen för skogens produkter
Uppsala 2013

Summary

In order to evaluate the possibilities of using enzymes for pre-treating softwood chips as a means of lowering the energy demand in mechanical pulping, impregnation and refining trials were performed using a set of different enzymes.

The enzymes tested pectinase, xylanase and mannanase gave increased sugar release in the impregnation trials indicating that most of the sugar released occurred in the first 60 minutes and that activities thereafter seemed to level off.

Refining trials using a small Wing refiner showed that for chips treated for 60 minutes with pectinase, xylanase and mannanase no energy savings to a given freeness level was observed. The property development was similar to that of reference pulps in the case of pectinase and xylanase while for chips treated with mannanase a less favourable development of the tensile index was noted.

For chips treated for two hours, using xylanase or pectinase, energy savings could be observed for pectinase treated chips down to a freeness level of 200 CSF. However when refined further, the properties approached those of the reference pulp.

Considering the much higher enzymatic activity reached when the initial fibre material was further disintegrated it is assumed that the possibilities for enzymes to attack desired structures of the intact fibre wall may have been too few even in the case of Impressafiner treated material.

Contents

Summary
Contents **3**
Background **4**
WOOD STRUCTURE 4
ENZYMATIC PRE-TREATMENTS 5
Experimental **6**
WOOD 6
ENZYMES 6
PILOT SCALE IMPREGNATION 6
REFINING 6
ANALYSIS 8
Results and discussion **9**
IMPREGNATION 9
REFINING TRIALS 9
PULP PROPERTIES 10
Conclusions **13**
Acknowledgements **14**
References **15**
Experimental **18**

APPENDIX 1. SMALL SCALE ENZYMATIC IMPREGNATION TRIALS
Silvia Viforr, Tomas Mårtensson and Lennart Salmén, Innventia, Stockholm

APPENDIX 2. PECTINASE ACTIVITY FOR TREATMENT OF SOFTWOOD CHIPS
Paul Ander and Geoffrey Daniel, SLU, Uppsala

APPENDIX 3. HISTOCHEMICAL ANALYSIS ON THE EFFECT OF PECTINASE PRE-TREATMENTS ON WOOD CHIPS FOR TMP REFINING
Dinesh Fernando and Geoffrey Daniel, SLU, Uppsala

Background

Thermomechanical pulping (TMP) is a highly energy demanding pulping process with an electrical energy consumption of usually more than 2,000 kWh/t. Due to the increasing energy costs as well as environmental considerations there is a high demand for ways of making the process more energy efficient. One approach is to modify the raw material, i.e. wood chips, prior to refining by mechanical chemical or enzymatic means. In this study pre-treatment using different types of enzymes has been evaluated.

Enzymes are complex proteins that have specificity for targeting particular molecular structures and are effective catalysts in biochemical processes even in low dosages. It is well known that a weakening of the outer cell wall structures, i.e. of the primary cell wall, by chemical means results in improved fibre separation and lower energy demand in refining. Thus enzymes that could specifically attack the structures of the primary cell wall would be of particular interest for evaluation. Here treatment with different pectinases, a xylanase and a mannanase have been evaluated.

WOOD STRUCTURE

For producing a good thermomechanical pulp at reasonable energy demand, it is essential that a high degree of intact fibres can be separated from chips and that these fibres that can be easily further refined. Thus the fibre fracture should preferably take part in the outer fibre wall layers (Franzén 1986) in the primary wall/S_1 region. Here the properties of the primary wall are of particular interest as its composition varies considerably from that of the other cell wall layers. The primary wall being reminiscent from the living cell wall is constituted, apart from disordered cellulose, of xyloglucans, pectins, proteins and lignin with multiple interactions between the components, as illustrated in Figure 1 (Stevanic Srndovic 2011). Attacking the primary cell wall specifically would therefore be of advantage. It has earlier been demonstrated that with the use of low chemical dosages, specifically with bisulphite, fractures in the primary wall occur leading to lower energy demand in subsequent refining (Axelson and Simonson 1982a, b; 1983a, b; Westermark et al. 1987).

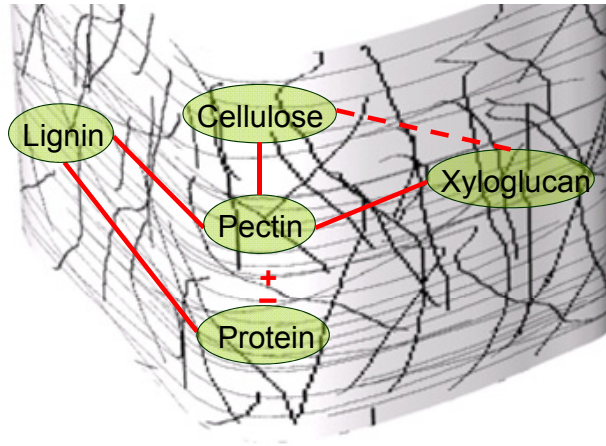


Figure 1. Schematic diagram of the interactions between the polymer components of the primary cell wall (Stevanic Srndovic 2011).

ENZYMATIC PRE-TREATMENTS

It was not until rather recently that the introduction of enzymes as a pre-treatment of wood chips was studied (for example see: Richardson et al. 1998; Peng et al. 2003). Since then, tests at both laboratory and mill scale have been performed but relatively few reports have been published on the pre-treatment of wood chips and results from experiments suggest a non-conformity regarding the possibility of achieving reductions in energy consumption during refining. In many cases energy reductions have been documented at a specific freeness level. However when comparing pulps at similar tensile strength, the situation is variable and often no energy savings have been reported.

Pre-treatments using cellulases have been shown to result in large energy savings using Wing-refiners (Pere et al. 2005). However, such energy savings have so far not been possible to reproduce in conventional refiners other than as energy savings to a specific freeness level (Salmén 2008). Xylanases have been shown to give large energy savings when treating poplar; up to 25% (Petit-Conil 2005). In the case of treatment of spruce chips no significant impact on the energy-property relationship has been noted (Petit-Conil 2005). Treatments using pectinases have shown a 10% energy reduction for spruce samples above 100 ml freeness, but only marginal effects when it comes to comparisons at a given strength level (Peng et al. 2003). A similar case is observed for proteinases with the breaking point in freeness being 250 ml (Mansfield et al. 1999).

Experimental

WOOD

The wood material used was Norway spruce chips supplied by Braviken Mill, Holmen Paper. The chips had been treated previously in an Impressafiner at Braviken in order to break up the chip/wood structure. In this treatment, chips are pre-steamed and thereafter compressed and sheared through the Impressafiner. The treated chips were delivered to Innventia and frozen for storage. Prior to the trials the chips were thawed (two weeks before trials).

ENZYMES

The enzymes used were: multipectinase (NS81272), xylanase (NS51115) and mannanase (NS51054) all supplied by Novozymes. The recommended dosages were 1 kg of enzyme/ton DMC (dry matter content) wood. The optimal conditions for these enzymes were 50 °C and pH 5.0 for pectinases, 55 °C and pH 5.5 for xylanase, and 80 °C pH 5.0 for mannanase. Xylanase was reported to have minor cellulase background activity and major endo-1,4 xylanase activity.

For adjusting the desired pH values, buffers of sodium acetate were prepared by addition of glacial acetic acid mixed with sodium acetate trihydrate and diluted with dH_2O to pH 5.0 (pectinase and the mannanase) and 5.5 (xylanase) (0.1 M).

PILOT SCALE IMPREGNATION

Pilot scale impregnations of wood chips were made using a PREX screw connected to a larger storage tank at Innventia. The tank of approx. 400 l was filled with 200 l dH_2O adjusted to a temperature of 43-48 °C. Enzyme solutions (1.5; 2.0; 2.0 kg enzyme product/ton dry wood of pectinase, mannanase, and xylanase respectively) were added to the water and stirred. Ten kg of Impressafiner wood chips was first steamed for 10 minutes and fed into the tank by a rotating screw. The suspension in the tank was stirred half way through feeding. When all the wood chips had been fed into the tank the solution was again stirred. Incubation of wood chips was continued for 60 minutes where after half of the chips were removed (excessive water was put back in the tank before the wood chips were transferred to another container). The collected wood chips were steamed for 10 minutes to stop any enzymatic activity, and the wood material was then put in a refrigerator. The remaining chips were incubated for another 60 minutes and the above handling of the wood material repeated.

REFINING

Refining was made using a Wing-refiner either at Aalto University in Helsinki (Figure 2), for treatments of 1 hour or at SCA, Sundsvall for treatments of 2 hours.

At Aalto University the impregnated wood chips were portioned into 125 g batches (dry matter content) and refined in a low-intensity wing refiner. The wing defibrator chamber consisted of a 20 counter blade cylinder with a distance of 1 mm from 4 wing-like rotating blades to the counter blades. Three empty runs of the steam heat treated refiner were used as a blank. Chips were steam treated at a temperature of 124 °C ± 0.6 °C for 5 minutes, during which the 4 wing-like blades were rotated 90° every 1.25 minutes to heat the chips evenly. After 2 minutes steaming the condensate was released during 10 seconds. After 4 minutes and 50 seconds of steaming the valve was closed, the pulse-meter zeroed and the run started after 5 minutes of steaming. Refining was performed for 2, 4, 6 and 8 minutes respectively. The pressure in the chamber during refining was 1.9-2.6 bars with the temperature rising from about 124 °C to 136 °C; the temperature depending on how long the experiment was continued. All enzyme trials were run singular, as well as trials at 2, 6, and 8 minutes for the reference. Four minutes of refining for the reference was run 4 times. Unrefined/less refined wood chips/pulp between the wing refiner body and the wing refiner cap was removed. Small pieces of unrefined wood chips were observed inside the wing refiner.

Figure 2. Wing refiner at Aalto University in Helsinki.

At SCA the refiner housing of the Wing-refiner was heated to the desired temperature. The chips were inserted in the refiner and live steam used for pressurizing the refiner and also for pre-steaming the chips. The chips were atmospherically steamed for 300 seconds with the hatch partly closed. The hatch was then closed and the chips were preheated with live steam for 3 minutes at 0.5 bar. Water was added to reach the calculated refining consistency of 30%.

Refining was carried out at 1200 rpm to the desired energy input giving different retention times in the refiner. When the desired energy input was obtained the refiner was stopped and the pressure released. The hatch was then opened and the pulp was taken out of the refiner and put into a bucket.

The pulp was subsequently hot disintegrated and screened in a Wennberg screen over a 0,15 mm slotted screen plate.

ANALYSIS

Enzyme activity during impregnation was determined using the Nelson-Somogyi method which measures the amount of reducing sugars (i.e. sugars that contain aldehyde groups that are oxidized to carboxylic acids) using a spectrophotometer (Farnet 2010, Sadasivam 2007, Wrolstad 2001).

Pulp testing was made according to SCAN-M 4:65 (Freeness), ISO 5263-3:2004 (Hot disintegration), ISO 5269-1:2005 (Laboratory sheets), ISO 5270:1999 (Testing of lab. sheets), ISO 536:1995 (Basis weight), SCAN-P 88:01 (Structural density), ISO1924-3:2005 (Tensile index), ISO 9416:2009 (Light scattering), L&WFiber-Tester (Fibre length).

Results and discussion

IMPREGNATION

The pilot scale impregnation was performed using PREX equipment. For the enzymes pectinase and xylanase, samples were prepared with both 60 and 120 mins incubation while for mannanase only incubation for 60 minutes was performed.

The amount of released sugar increased with time up to 60 minutes for all samples including the reference. Much of the released sugars seemed to remain in the chip structure making it is difficult to follow the process during treatment. When the liquid was pressed out from the treated chips, clear enzymatic activity was apparent for pectinase, xylanase and mannanase (see Figure 3; 60 minutes incubation).

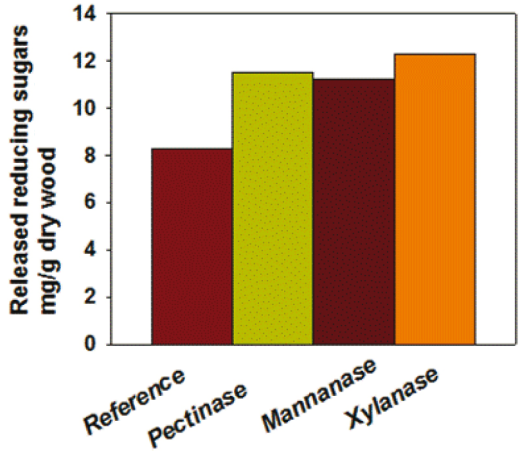


Figure 3. Amount of released reducing sugars from different enzyme treatments of Impressafiner chips (total amount based on concentration in pressed-out liquid). Incubation time 60 minutes.

REFINING TRIALS

Refining experiments were carried out on the enzymatic treatments with pectinase, xylanase and mannanase performed for 60 minutes in a Wing-refiner at Aalto University in Helsinki, Finland and for the enzymatic treatments with pectinase and xylanase performed for 120 minutes in a Wing-refiner at SCA, Sundsvall.

In Figures 4 and 5, the freeness development for trials is summarized. Evidently no significant effect of the different enzyme treatments could be noted regarding the freeness level for treatments after one hour while for the two hour treatment, a

slightly reduced energy demand to a specific freeness level was shown. The two refining trials at the different facilities of Aalto University and SCA produced pulps with very similar freeness/energy relationships.

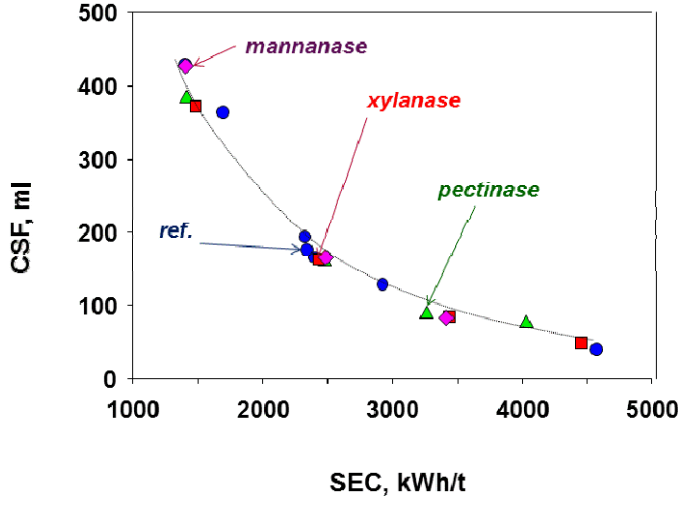


Figure 4. Freeness versus energy consumption for Wing-refining of batches of Impressafiner chips pre-treated with different enzymes for 60 minutes.

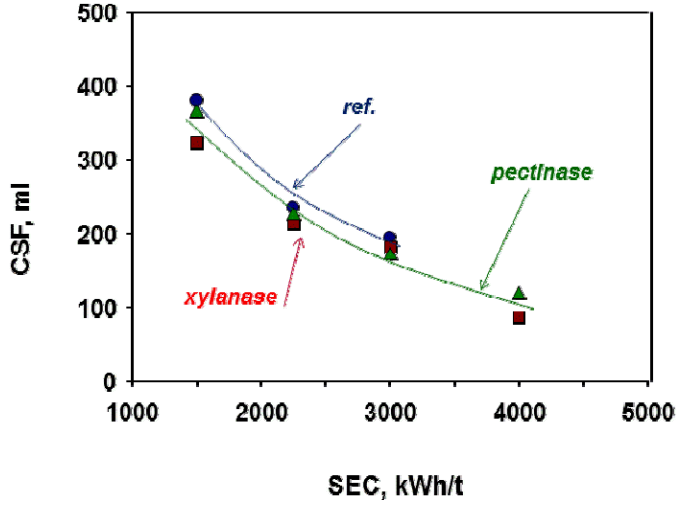


Figure 5. Freeness versus energy consumption for Wing-refining of batches of Impressafiner chips pre-treated with different enzymes for 120 minutes.

PULP PROPERTIES

Pulp properties were evaluated for pulps from chips treated for 1 hour having a

freeness level between 200 and 85 in CSF and for pulps from the 2 hour treated chips in the freeness interval of 400 to 100 CSF. Prior to paper making, the shives were removed in a Metso screen. As seen in Figure 6, none of the enzyme pre-treatments for one hour treated chips resulted in improved strength properties with respect to the energy consumption. In the case of mannanase treatment, pulp refining to the low freeness level seemed to cause development in a less favourable manner compared to the other treatments. This may be a consequence of mannanase treatment not being specifically targeted to the primary wall region. For chips treated for 2 hours, pectinase treatment resulted in improved strength properties at moderate energy input levels, i.e. above a freeness level of 200 CSF. Here an energy saving of ca 300 kWh/ton was estimated. However, when refined further to lower freeness levels the difference between the pectinase treated pulp and the reference treated pulp disappeared. A similar development with refining was also earlier observed for pectinase treated samples in pilot plant refining (Peng et al. 2003). The difference in paper properties between the two different batches of enzyme treatments mainly reflects the different procedures used at Innventia for 1 hour treated chips compared to those prepared at Stora Enso Karlstad for the 2 hour treated chips, the former having a higher density.

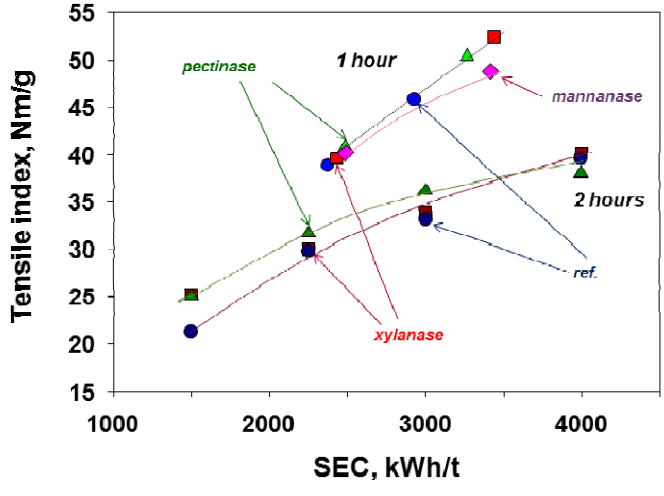


Figure 6. Tensile index versus energy consumption for Wing-refining of batches of Impressafiner chips pre-treated with different enzymes for either 60 or 120 minutes.

Light scattering developed in a similar manner for all the pulps (Figure 7). The test sheets prepared at Stora Enso from pulps of chips treated for two hours had in general a somewhat lower density than sheets prepared at Innventia from the 1 hour treated chips as reflected in a higher light scattering at similar tensile index. The only tendency observed may be that the pectinase treated samples have a lower light scattering at comparable tensile index.

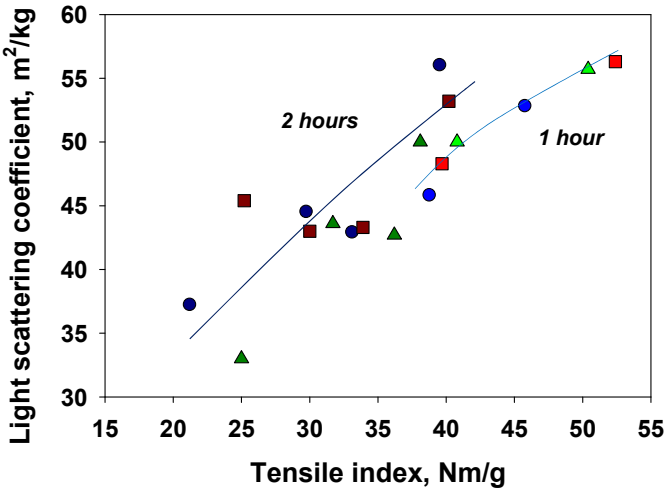


Figure 7. Light scattering coefficient versus tensile index for Wing-refining of batches of Impressafiner chips pre-treated with different enzymes for either 60 or 120 minutes. Circles – reference pulp; triangles – pectinase; squares – xylanase.

Conclusions

Impregnation of Impressafiner treated chips with enzyme solutions were shown to result in increased degradation of the fibre material as indicated by an increased dissolution of degraded carbohydrates.

With refining of such impregnated chips, no energy savings as a function of freeness level was observed for chips treated for one hour with pectinase, xylanase or mannanase. Property development was similar to that of reference pulps in the case of pectinase and xylanase while for chips treated with mannanase a less favourable development of the tensile index was noted.

For chips treated for two hours an energy saving could be observed for pectinase treated chips down to a freeness level of 200 CSF. However, when refined further the properties approached those of the reference pulp. Considering the much higher enzyme activity reached when the initial fibre material was further disintegrated, it is assumed that the possibilities for enzymes to attack desired structures of the intact fibre wall may have been too limited even in the case of the Impressafiner treated material.

Acknowledgements

The supply of enzymes by Novozymes A/S and the technical advice regarding their use by Kasper Klausen of Novozymes is greatly appreciated by the authors.

Aalto University and Prof. Herbert Sixta is acknowledged for their contribution regarding the use of the Wing-refining facilities at the Department of Forest Products Technology. Mr Timo Ylönen of the Department is also greatly acknowledged for his technical advice and help in running the refining experiments.

References

Axelson P and Simonson R (1982a). Thermomechanical pulping with low addition of sulphite. Influence of the preheating temperature at mild sulphite treatment of spruce chips prior to defibration. Paperi ja Puu 64(11), 729-733.

Axelson, P. and Simonson R (1982b). Thermomechaniocal pulping with low addition of sulfite. Part 1. Effects of mild sulfite treatment of spruce chips prior to defibration. Sv. Papperstidning 85(15), R132-139.

Axelson P and Simonson R (1983a). Thermomechanical pulping with low addition of sulfite. Part 3. Mild sulfite treatment of a mixture of spruce and pine chips. Sv. Papperstidning 86(3), R17-21.

Axelson P and Simonson R (1983b). Thermomechanical pulping with low addition of sulphite. Part 4 A mill scale trial. Sv. Papperstidning 86(15), R149-151.

Farnet A M, Qasemian L, Guiral D and Ferré E (2010). A modified method based on arsenomolybdate complex to quantify cellulase activities: Application to litters. Pedobiologia 53(2), 159-160.

Franzén R (1986). General and selective upgrading of mechanical pulps. Nordic Pulp Paper Res. J. 1(3), 4-13.

Mansfield S D, Won K K Y and Richardson J D (1999). Improvements in mechanical pulp processing with proteinase treatments. Appita J. 52(6), 436-440.

Peng F, Ferritsius R and Ängsås U (2003). Mechanical pulping with pectinase pretreatment of wood chips. 2003 International Mechanical Pulping Conference, Quebec. 335-340.

Pere J, Ellmen J and Viikari L (2005). Process for preparing mechanical pulp. Finland WO2005056915.

Petit-Conil M, Hoddenbagh J, Marc A, Tolan J S (2005). Method for mechanical pulp production. WO 2005/106110 A1.

Richardson J D, Wong K K Y and Clark T A (1998). Modification of mechanical pulp using carbohydrate-degrading enzymes. J. Pulp Paper Sci. 24(4), 125-129.

Sadasivam S and Manickam A (2007). Biochemical methods. New Age International (P) Ltd.

Salmén L (2008) Ecotarget. EU-project; Personal comunication

Stevanic Srndovic J (2011). Interaction between wood polymers in wood cell walls and cellulose/hemicellulose biocomposites. Ph.D. thesis. Chemical and Biological Engineering, Chalmers University of Technology. Gothenburg, Sweden

Westermark U, Samuelsson B, Simonson R and Pihl R (1987). Investigation of a selective sulfonation of wood chips. Part 5. Thermomechanical pulping with low addition of sulfite. Nordic Pulp Pap. Res. J. 2(4), 146-151.

Wrolstad R E (2001). Current protocols in food analytical chemistry. New York, Wiley.

APPENDIX 1. SMALL SCALE ENZYMATIC IMPREGNATION TRIALS

Silvia Viforr, Tomas Mårtenssson and Lennart Salmén, Innventia

Summary

In order to evaluate the possibilities of using enzymes for pre-treating softwood chips to lower the energy demand in mechanical pulping impregnation trials were performed using a set of different enzymes. In laboratory scale impregnation trials it was shown that the enzymatic activity highly depended on the size, i.e. the amount of destruction of the chips with more disintegrated wood chips rendering a higher release of sugars. Of the enzymes tested, pectinase and xylanase resulted in the highest amount of released sugars while mannanase and pectin lyase gave considerably lower sugar release.

Introduction

The main problem to use enzymes for pre-treatment of wood chips is the accessibility of the proteins for the desired structure of modification, i.e. the primary cell wall. In general, the size of enzymes are too large to access the cell wall of intact chips and access may only be gained via ray cells and borded pits into the middle lamella/primary wall region. Thus a mechanical destruction of the chips provided with Impressafining is highly recommended for increasing the possibility of improved accessibility.

ENZYMES

In the studies performed, pectinases, xylanases, and mannanases were chosen based on their ability to attack the primary wall, availability and thermal stability (the latter property of importance for industrial processing of steam treated wood).

Pectinases

Pectinases can be divided into three main categories; protopectinases, esterases and depolymerases. The former degrade protopectin by adding water; esterases remove methoxyesters (-R-COOCH3 -R-COOH); and the latter cleaves α-1,4-bonds in galacturonic acid either by a hydrolytic mechanism, where a water molecule is introduced across the oxygen bridge, or by trans-elimination lysis where water molecules are not needed to break the glycoside bond. Pectin lyases by themselves can degrade pectin, whereas pectinesterases and polygalacturonases must co-work to completely degrade pectin. These reactions are illustrated in Figure 1 (a)-(b).

Xylanases

Due to the complexity of xylan, with different side groups there are no definite reactions for this class of enzyme. A typical reaction can be seen in Figure 1 (c).

Manannases

Mannanases break down the bonding between mannan units in glucomannans. A general reaction is shown in Figure 1 (d).

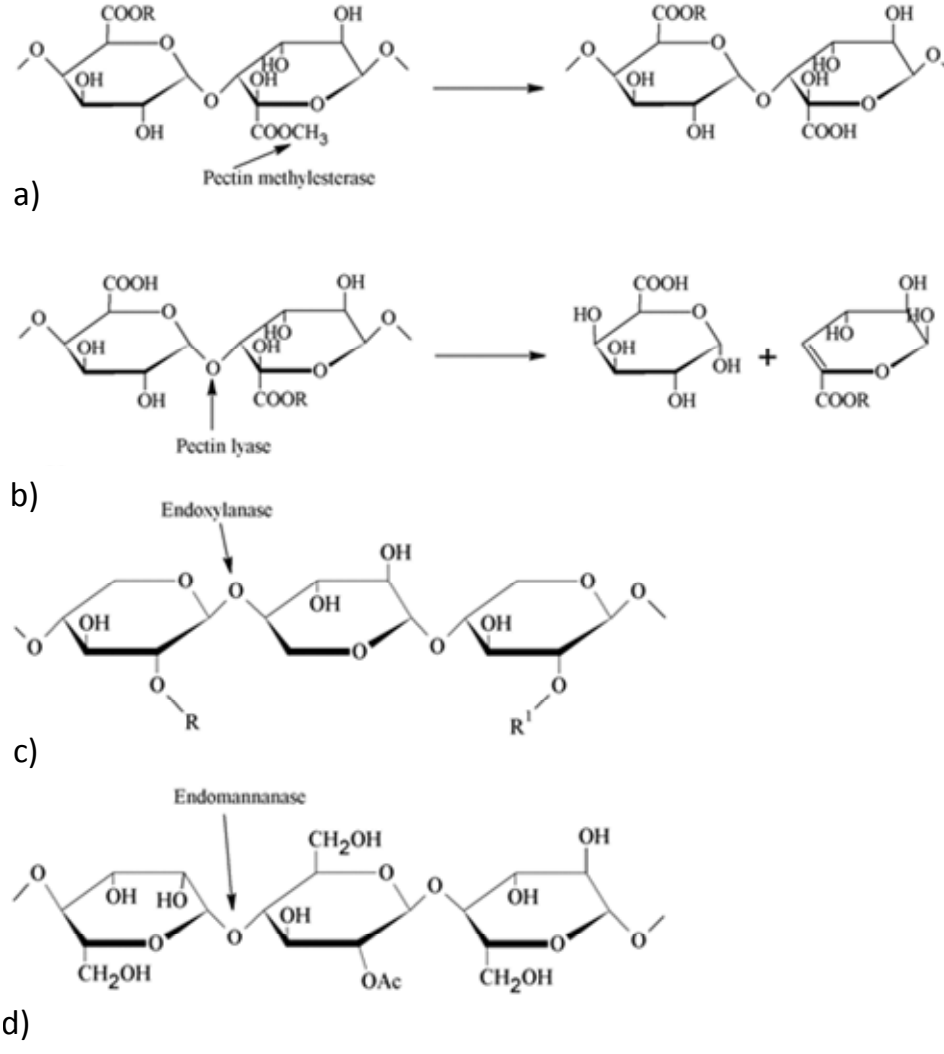


Figure 1. Wood substrates and reaction of some enzymes (a)-(b) pectin (R = CH3 or H) (pectin lyase and pectin methylesterase), (c) xylan (R and R1 = Arabinose, other polysaccharides, etc.) (endoxylanase), and (d) mannan (endomannanase).

Experimental

WOOD

The wood material used was Norway spruce chips supplied by Braviken Mill, Holmen Paper. The chips had been previously treated in an Impressafiner at Braviken in order to break up the chip/wood structure. In this treatment, chips are pre-steamed and thereafter compressed and sheared through the Impressafiner. The treated chips were delivered to Innventia and frozen for storage. Prior to the trials, the chips were thawed (two weeks before trials). Wood shavings of Norway spruce chips were used for comparison. The wood materials are illustrated in Figure 2.

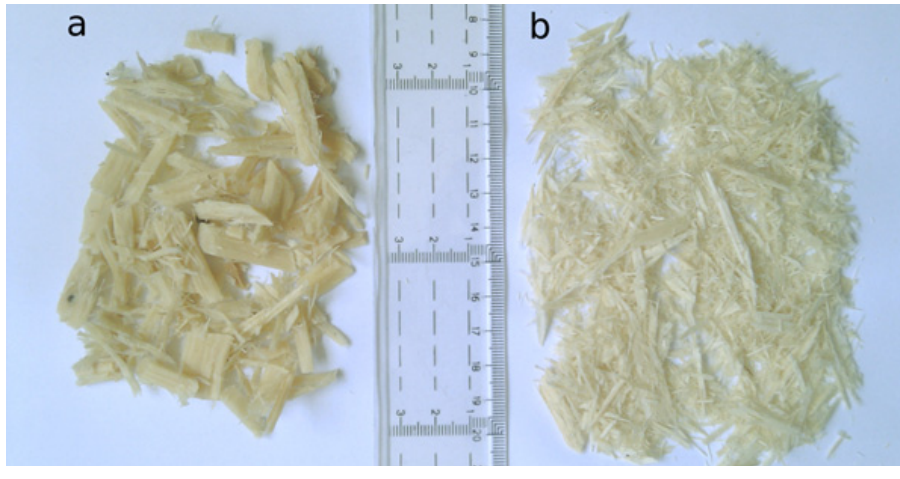


Figure 2. Visualization of the wood material of Norway spruce. a = Impressafiner chips, b = wood shavings.

ENZYMES

The enzymes used were: pectin lyase in combination with a pectin methylesterase (PME), multipectinase (NS81272), xylanase (NS51115) and mannanase (NS51054), all supplied by Novozymes. The recommended dosages were 1 kg of enzyme/ton DMC (dry matter content) wood, except for pectin methylesterase which was recommended at 0.1 kg/ton DMC (dry matter content) wood. The optimal conditions for these emzymes were 50 °C and pH 5.0 for all pectinases, 55 °C and pH 5.5 for xylanase and 80 °C pH 5.0 for mannanase. Xylanase had a minor cellulase background activity and major endo-1,4 xylanase activity.

For adjusting to the desired pH values, buffers of sodium acetate were prepared by addition of glacial acetic acid mixed with sodium acetate trihydrate and diluted with dH_2O to pH 5.0 (pectinase and mannanase), 5.1 (pectin lyase/pectin methylesterase) and 5.5 (xylanase) (0.1 M).

Laboratory enzyme treatments

In order to impregnate the wood material with enzymes as well as perform treatments in laboratory scale, an autoclave was used allowing for compression of steam treated chips. Steam and enzymatic solutions were applied from the top connections, while sampling of solution was made from the bottom tap. The chips inside the autoclave may be manually compressed by a top screw.

Spruce chips (64 g DMC of Impressafiner chips, 46g DMC of spruce shavings) were steamed for 5 minutes (1 bar) and compressed to 50% of the height of the filled container. Thirty ml of 0.1M sodium acetate buffer was added (pH 5.5) for xylanase, pH 5.1 for pectin lyase/pectin methylesterase and pH 5.0 for pectinase and mannanase). The apparatus was moved to a water bath, maintained at approximately 18 °C, for 10 minutes during which 200 ml buffer was added. When mannanase was tested, the apparatus was cooled at room temperature for 5 minutes, reaching a temperature of 79 °C. The apparatus was emptied of liquid and moved to a water bath (80 °C for mannanase, 50 °C for all pectinases, and 55 °C for xylanase) for 5 minutes. Thirty ml of enzyme solution was added, the mechanical press was released, the cap was substituted to the foot of an E-flask, the wood chips were loosened with a spoon and the rest of the enzyme solution was poured onto the wood. Time was taken when all the enzyme solution had been added. During incubation, samples of the solution were taken every 10 minutes. After incubation for 90 minutes, the solution was drained and collected. The wood chips were then steamed for 5 minutes with the bottom tap open, during which the drained solution was collected. The wood chips were again compressed (approximately 50%) to remove some of the remaining liquid. The procedure is outlined in Figure 3.

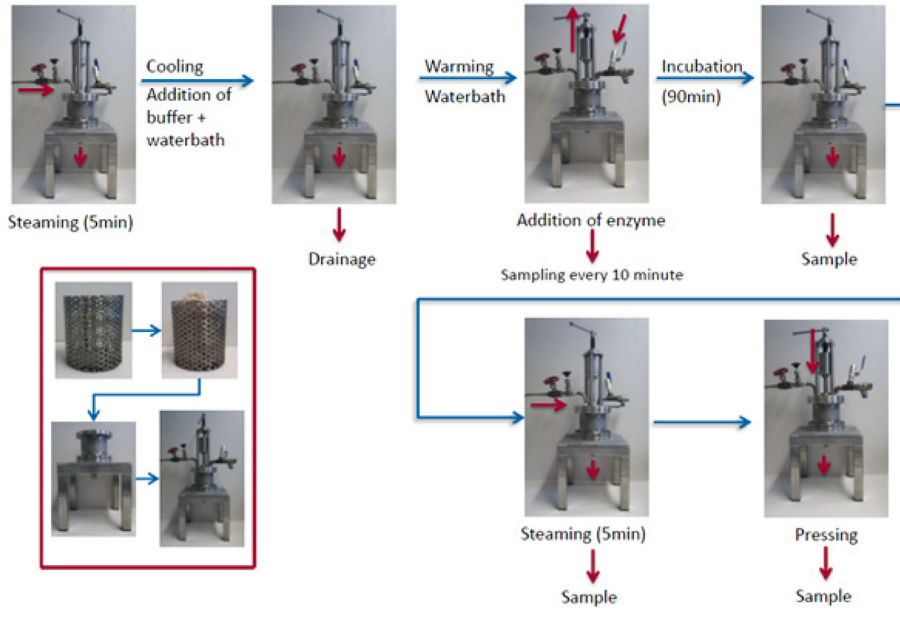


Figure 3. Visualization of wood chip pre-treatment in laboratory trials. The perforated container in the lower left corner was covered in a 200 mesh (74µm) net.

Tests of Enzyme activity

The effect of the enzyme activity in the experiments was determined by using the Nelson-Somogyi method which measures the amount of reducing sugars, i.e. sugars that contain aldehyde groups that are oxidised to carboxylic acids. The analysis is based on the reduction of Copper (Cu) (II) ions to Cu (I) ions by a saccharide molecule, e.g. D-glucose, D-galactose, maltose, etc., which in this case is released after enzymatic hydrolysis of the saccharide chains in the wood materials used. The Cu (I) ions are thereafter oxidised back to Cu (II) by a colourless arseno(poly)molybdate complex, which in a reduced form is blue. Absorption at 500 or 520 nm is then measured using a spectrophotometer (Farnet 2010, Sadasivam 2007, Wrolstad 2001).

Results

The wood material had a dry matter content of 36.5% for the Impressafiner wood chips and 59.5% for the finer wood chips.

In the laboratory autoclave the compression after the initial steam treatment increased the dry matter content to 39.5% for the Impressafiner wood chips while after absorption of the enzyme solution the dry matter content was reduced to 34.5%.

The enzymatic pre-trials performed are summarized in Table 1.

Table 1. Summary of enzyme laboratory pre-treatments of spruce chips and raw materials

Enzyme	Dosage (kg/tondry wood)	Wood	∑(Reducing Sugars) (µg/g dry wood)	Sugars Enzyme / Reference
Pectin lyase + PME	1.5 + 0.5	Impressafiner	490	1.3
Pectin lyase + PME	5 + 1	Impressafiner	370	1.0
Pectinase	1	Impressafiner	540	1.5
Reference Pect. impr. a)		Impressafiner	300	
Reference Pect. imp. b)		Impressafiner	430	
				Average 365 µg/g
Pectin lyase + PME	1.5 + 0.5	Fine chips	670	1.5
Pectinase	10	Fine chips	1610	3.7
Reference Pect. fine a)		Fine chips	450	
				Average 435 µg/g
Reference Pect. fine b)		Fine chips	420	
Mannanase	1.5	Impressafiner	650	1.2
Mannanase	5	Impressafiner	710	1.2
Reference Man.		Impressafiner	570	
Xylanase	1,5	Impressafiner	660	1.5
Reference Xyl.		Impressafiner	440	

It was clearly evident that the structure of the wood chips had a large impact on the accessibility of the enzymes to the wood polymers. Thus much higher amounts of released sugars were obtained from the finely disintegrated wood chips than from the impressafiner treated chips even though these had a rather disrupted structure. As shown in Figure 4, the amount of released sugars from the finer chips (i.e. shavings) was nearly doubled in the case of the enzymes pectin lyase/PME compared with impressafiner chips.

For pectin lyase/PME the use of 1.5 times the recommended amount of enzyme only showed a slightly higher degree of degraded (reducing) sugars compared to the reference. No improvement was seen when increasing the dosage to 5 + 1 kg/ ton of dry wood (Table 1). With the Impressafiner wood chips, pectinase and xylanase resulted in the highest amount of released sugars. For mannanase, use of the recommended dose of 1.5 kg/ton dry wood did not result in much of an increase in reduced sugar release and increasing the amount to 5 kg/ton dry wood only slightly increased the amount of released sugars.

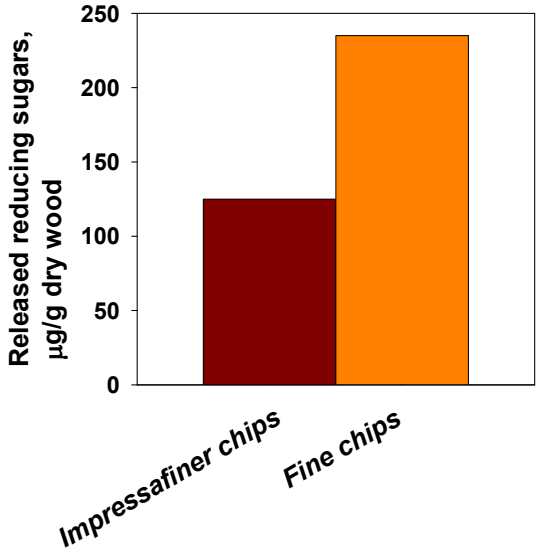


Figure 4. Amount of released reducing sugars from different types of chips using a mixture of enzymes, Pectin lyase/PME in a quantity of 1.5/0.5 kg/ton.

Conclusions

Based on these measurements it was decided to perform large scale experiments using pectinase and xylanase. Also an additional test using mannanase was included despite the low amount of sugars released but based on the high temperature tolerance (i.e. 80 °C) which from a process consideration is of interest.

References

Farnet A M, Qasemian L, Guiral D and Ferré E (2010). A modified method based on arsenomolybdate complex to quantify cellulase activities: Application to litters. Pedobiologia 53(2), 159-160.

Sadasivam S and Manickam A (2007). Biochemical methods. New Age International (P) Ltd.

Wrolstad R E (2001). Current protocols in food analytical chemistry. New York, Wiley.

APPENDIX 2. PECTINASE ACTIVITY FOR TREATMENT OF SOFTWOOD CHIPS

Paul Ander, Geoffrey Daniel, SLU, Uppsala

Background

Pretreatment of spruce wood chips with different pectinases and other enzymes were investigated in order to decrease refining energy during TMP pulping (Peng et al. 2003; Månsson 2012). In this connection it is important to evaluate the pectinase activity to be used. Here pectinase activity against polygalacturonic acid was used as a model assay for comparison of two types of pectinases and relate their activity to similar activity against Impressafiner spruce chips and wood shavings.

EXPERIMENTAL

Enzymes

Pectin lyase and multipectinase (NZ863) were both from Novozymes, Denmark. Protein content was measured using the relation absorbance at 280 vs 260 nm (Augustinsson 1966). Original protein content was 9.1 mg/ml for Smash and 15-20 mg/ml for Ultra. The pectinases were desalted on Sephadex PD-10 G-25 columns (Amersham Biosciences, Uppsala) to remove salts, monosugars and inhibitors. Dilution factor was 1.4. The purity of the Pectin lyase and multipectinase were tested by electrophoresis on SDS-PAGE gels (Pharmacia, Uppsala). There was a dominant band at ca 45000 kda for both enzymes representing the active pectinase. The multipectinex NZ863 had a more complete protein profile (Figure 1). For both enzymes a smaller band just below 70000 kDa was found.

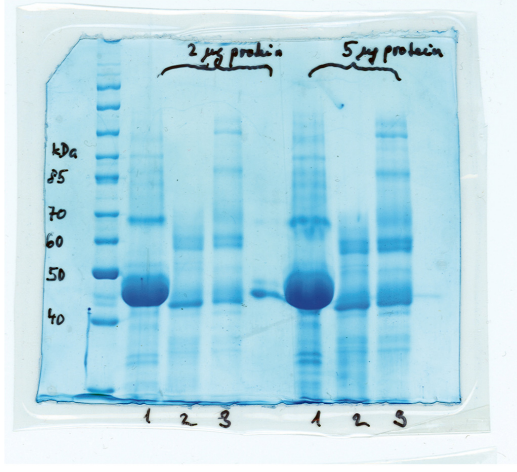


Figure 1. Electrophoresis of used pectinases: 1) multipectinase NZ863; 2) pectine lyase; and 3) visozyme. The lane to the left shows the reference proteins (molecular weight markers).

Substrate
Polygalacturonic acid (P3889, PGUA) was from Sigma-Aldrich.

Pectinase assay
Four to five ml acetic acid buffer pH 5 and 50 mg PGUA were mixed with a Super Mixer in test tubes and desalted pectinases added and mixed. Incubation in a total volume 5 ml was at 50 °C for 30 min, with mixing of the PGUA-suspension after 15 min and 30 min. The suspension was then allowed to sediment for 2h in the cold room.

Assay of reducing sugars from PGUA
From the supernatant 0.2 ml samples were taken for reducing sugar determination by the BCA method (Garcia et al. 1993). The samples were diluted 30x, and incubated in the BCA assay for 30 min at 80 °C. Absorbance at 560 nm gives relative reducing sugar formation from the two pectinases. A typical glucose standard curve is shown in Figure 2.

RESULTS
The results of the pectinase assays are shown for Pectin lyase in Figures 3 and 4 and for multipectinase (NZ863) in Figure 5. The substrate saturation point for Smash was assumed to be at about 1 ml enzyme, and this value was used for calculation of the amount of sugar. Multipectinase (NZ863) as seen in Figure 5 was stronger and the substrate was saturated already at 0.2 ml enzyme. As mentioned in Materials and Methods, pectin lyase had a lower protein content than multipectinase.

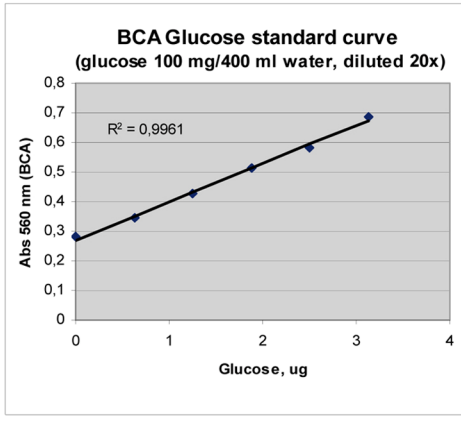


Figure 2. Typical glucose standard curve.

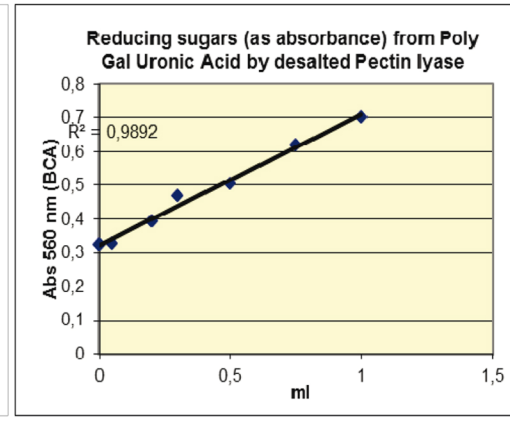


Figure 3. Release of reducing sugars PGUA by Pectin lyase. Assay nr 1.

Calculations. Smash Assays 1 and 2 gave 3.3-5.85 µg reducing sugars per 50 mg PGUA. Taking into account the dilution factor 1.4 obtained by desalting, the mean value for the two assays was 6.4 µg sugar. The resulting value for the multipectinase was 35 µg sugars. This means that multipectinase was more active and should be better for use than pectin lyase. Other results for Impressafiner spruce chips and for wood shavings in Appendix 1 indicate a similar result as shown in Table 1 on page 20. For example at a certain enzyme amount given in the upper part of Table 1, Pectin lyase + PME (pectin methylesterase) gave 370-490 µg reducing sugars per gram Impressafiner chips, while multipectinase gave 540 µg (0.54 mg) sugars per g chips.

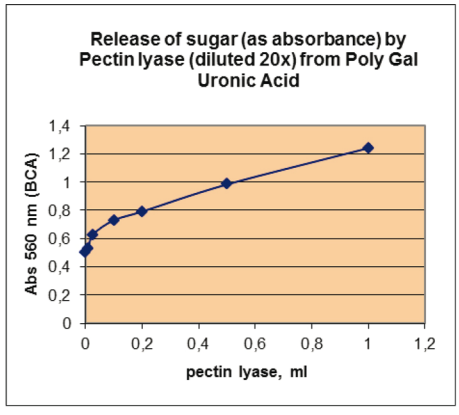


Figure 4. Release of reducing sugars from PGUA by pectin lyase. Assay nr 2.

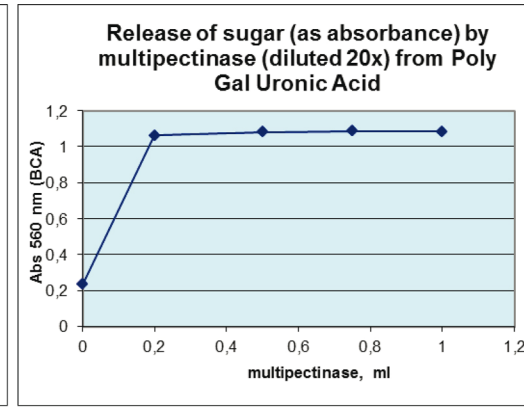


Figure 5. Release of reducing sugars from PGUA by multipectinase.

On page 8 of the Summary, Figure 3 shows that multipectinase gave almost 12 mg reducing sugars per gram Impressafiner chips treated for 60 min. A number of factors such as type of substrate, enzyme amount and treatment time may give such different values as 0.54 and 12 mg sugars. However, the results here indicate that 128 µg reducing sugars per gram PGUA can be obtained by pectin lyase and 700 µg sugars by multipectinase. This is partly of the same order as obtained using Impressafiner chips according to the values given above for multipectinase. Pectin lyase + PME may result in stronger sugar release than without PME.

CONCLUSIONS

The multipectinase released more reducing sugars from polygalacturonic acid (PGUA) than pectin lyase. A similar result was also obtained with Impressafiner spruce chips and wood shavings as shown in Appendix 1. Comparing that trial, the release of reducing sugar from both PGUA and Impressafiner chips were of the same order, namely 540-700 µg sugars per gram substrate. This indicates that PGUA is a suitable substrate to use in evaluation of pectinase activities for treatment of at least Impressafiner chips or wood shavings. However, one should not automatically expect that sugar yields from so different substrates such as PGUA and Impressafiner chips will be similar. A number of factors such as type of substrate, enzyme amount and treatment time etc., may give totally different values, especially for woody substrates.

References

Augustinsson, K-B (1966) Experimentell biokemi. Scandinavian University Books, Svenska Bokförlaget Bonnier, p. 361-362.

Garcia, E., D. Jonston, J.R Whitaker and S. Shoemaker (1993) Assessment of endo-1,4-beta-D-glucanase activity by a rapid colorimetric assay using disodium 2,2'-bicinchoninate. J. Food Biochem. 17, 135-143.

Mårtensson, T. (2012) Application of enzymes for pre-treatment of wood chips for energy efficient thermomechanical pulping. Examensarbete, Linköpings Universitet & Innventia.

Peng, F., Ferritsius, R. and Ängsås, U. (2003) Mechanical pulping with pectinase pretreatment of wood chips. 2003 Int. Mechanical Pulping Conf., Montreal, Canada. ISBN 1-896742-86-6, p. 335-340.

APPENDIX 3. HISTOCHEMICAL ANALYSIS ON THE EFFECT OF PECTINASE PRE-TREATMENTS ON WOOD CHIPS FOR TMP REFINING

Dinesh Fernando, Geoffrey Daniel, SLU, Uppsala

Introduction

Due to the accelerating cost of energy consumption in mechanical pulping, extensive research has been carried out during recent decades for finding ways for energy-efficient pulping processes. Among ways for modifying refining processes, the pre-treatment of raw materials (wood chips) have been considered by means of mechanical (e.g. screw press Impressafiner), chemical (e.g. sulphites) or enzymatic (e.g. pectinases, xylanases) treatments (Peng et al. 2003; Naithani el al. 2004; Petit-Conil 2005; Nelsson et al. 2011). However, there are few studies that have targeted on pre-treating wood chips with enzymes prior to refining as it is thought there is an inefficient ability of enzymes to penetrate inside the almost closed and intact structure of wood and wood fibre cell walls. In addition, there are few studies on the mechanism of action of enzymes within the wood structure which could render basic understanding of enzymatic pre-treatment and thereby provide clues for better utilization of enzymes during refining. Therefore localization and visualization of the effects of pectinase pre-treatment on wood chips was the major objective of this part of the project that would enable an improved understanding of enzyme activity with the intact wood cell structure.

Pectins are non-cellulosic acidic polysaccharides that are found primarily in the primary cell walls of plant cells with much lesser degree in secondary walls. The basic component of pectin is galactopyranosyl uronic acid residues (galactouronan) which are either methylesterified or non-esterified in the cell wall. Both acidic- and esterified pectins are present in the middle lamella (ML) regions of lignified mature wood fibres, parenchyma cells (ray- and epithelial cells) and pit-membranes of softwood fibres (Hafrèn et al. 2000). Histological techniques in cell biology have been applied in wood tissues for localizing and visualizing pectin distribution within the cell structure of wood samples. Ruthenium red, for example, has frequently been used as a standard pectin stain in plant/wood tissues and stains acidic pectins red/pink (Sterling 1970; Harsveld van der Veen and Van den Ent 1994).

Experimental

This was a preliminary study to investigate possible effects of pectinase treatment on the cell structure of spruce wood chips which have already been subjected to mild mechanical treatment using the Impressafiner to open up the native wood structure.

Therefore only wood samples that were subjected to one hour enzymatic treatment were used in the microscopy analyses. The reference samples (one hour under the same buffer but without the enzyme pectinase) were used for comparison.

Thin sections (few microns) were taken from the outside of wood chips (2x4 cm; both enzyme treated and non-treated) using a razor blade. The outer surfaces (i.e. peripheral regions of chips) of the wood blocks should have the greatest possibility for contact with the enzyme treatments providing optimum reactivity with the pectin present on the outer surfaces within the cell structure of the wood samples. Sections were placed in 0.05% aqueous ruthenium red solution for 5-10 min, the excess solution removed and mounted in glycerol before examination using a Leica DMLB light microscope (LM). Images were recorded digitally with a Leica DC 300 CCD camera.

Results and discussion

Reference wood samples showed characteristic red/pink staining pattern within the cell structure of mature softwood tissue. Staining was clearly visible within the middle lamella (ML) regions of fibers while the greatest staining intensity was given by cell corner ML indicating the presence of pectins with high concentrations (Figure 1). This means that fiber separation could easily be achieved during refining with less energy by pre-treating pectin (either partial removal or structural changes to pectin in the wall should disturb the integrity of fibers in the wood tissue) in the ML region.

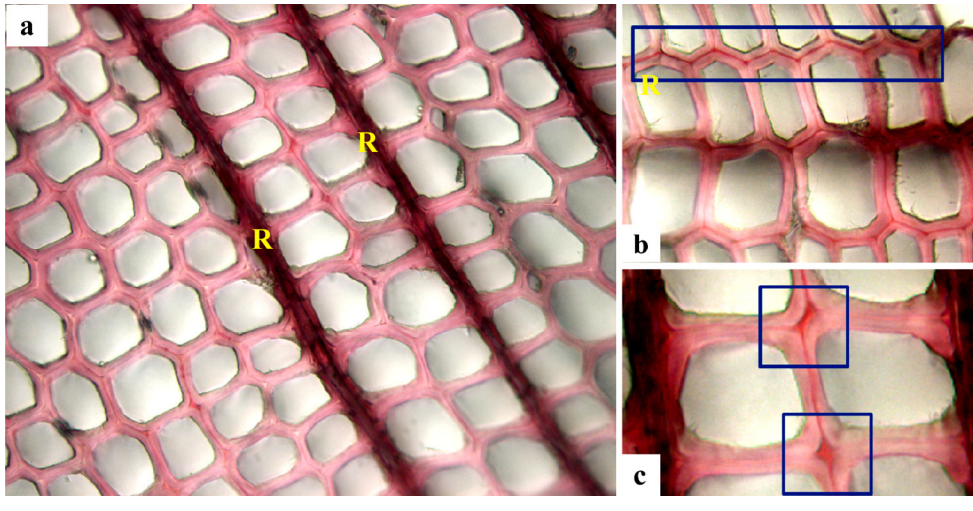


Figure 1a-c. Transverse sections of reference chip samples after ruthenium red staining. Strong intensity of red/pink is visible along rays (R in a), in ML regions (blue rectangle in b) and in the cell corner ML (blue squares in c).

In addition, ray parenchyma cell walls stained with RR (all rays were red/pink, Figure 2a) as they are also rich in pectins, particularly the CML (arrows in 2b) (Hafrén et al. 2000). Attack of pectins in rays should weaken the overall structural integrity of the cells of wood chips which in turn should influence energy demand during the initial phase of the refining process. Walls of parenchyma cells lining resin canals (i.e. epithelial cells; arrows in 2c, d) also stained with RR sometimes with less intensity compared to ray parenchyma. Pit membranes of bordered-pits of fibers were also stained red/pink (arrows in 2e and inset) although it was inconsistent over a given area of the sample. It was also found that reference samples sometimes showed heterogeneous staining reaction where either one or more cell types exhibited very weak red/pink colouration. This may be explained by the long incubation period of reference samples with low pH buffer solution (without enzymes) which may also have some effect on pectin chemistry in the cell wall. However, the present results on reference wood chips followed that known concerning pectin distribution within matured wood of Norway spruce.

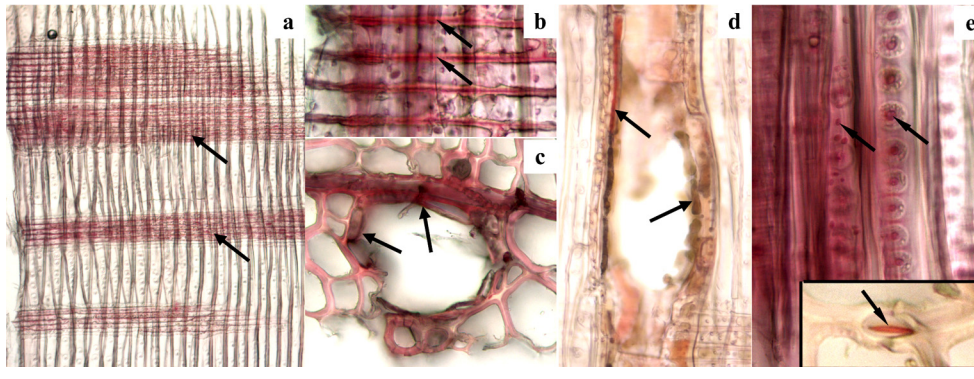


Figure 2a-e. Ruthenium red (RR) staining of reference wood samples showing typical spatial distribution of pectins: a) all rays in radial longitudinal (RL) sections stained red/pink (arrows) indicating the presence of pectins in ray parenchyma cells of a wood tissue; b) pectins are located in the ML regions of the cell wall of ray parenchyma (arrows, RL section); c) cell walls of epithelial cells lining resin canals stained irregularly with RR (arrows, transverse section (T) through a resin canal); d) RL section of a resin canal showing pectin distribution within epithelial cells (arrows); e) pectin presence in pit-membranes of bordered-pits (arrows) of Norway spruce (RL) was visualized after RR (inset, T section through a bordered-pit).

In contrast, pectinase treated samples showed either no staining or only pale red/pink colour after RR compared to reference samples (Figures 3-4). However, it should be noted here that results were irregular even within a sample sometimes with patches of weak staining. This indicates a non-homogeneous activity with the substrate although the latter should have the optimum possibility for better contact with the enzyme solution as the sections were taken from the outermost surfaces of

the wood chips. Figure 3 shows treated samples after staining and ML regions (i.e. CML) of the majority of fibers were not stained with RR. Instead the ML region sometimes appeared as white line over the sample (blue rectangle in 3b) probably indicating partial or complete removal of pectin due to pectinase activity. Whitish appearance was also observed in regions of the cell corner ML (arrows in 3c) where pectins were in high amounts in the reference wood. In addition, the majority of cells in treated samples were swollen (Figure 3a, d) with small lumen area due to the inward swelling of the inner secondary wall layers (arrows in 3d). This was predominant in latewood cells (Figure 3d) and the effect is most likely due to an indirect impact of pectin degradation process which opens-up the structure of intact native fiber cell wall. The swelling effect was rarely seen with reference samples (Figure 1a).

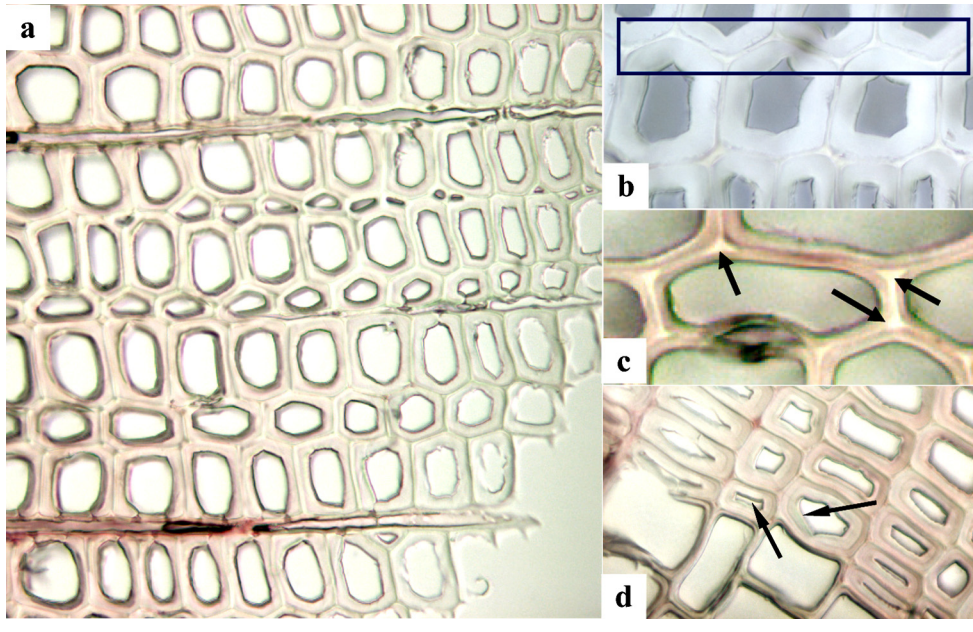


Figure 3a-d. Transverse sections of treated wood samples after RR staining: a) section through an earlywood part of the samples where fibers and rays showed either no- or very weak staining; b) blue rectangle to highlight the whitish line along the ML regions of some cells; c) cell corner ML (arrows) were commonly visible as white patches probably due to removal of pectins; d) latewood fibers were frequently swollen so that the cell lumen was very small (arrows) due to inward enlargement of cell walls towards the empty lumen.

The same result with signs of pectin degradation in treated samples was observed in rays (ray parenchyma, Figure 4a), epithelial cells lining resin canals (Figure 4b) and pit-membranes of bordered-pits of fibers (arrows in Figure 4c). Some epithelial cells also exhibited swelling by bulging outwards into the empty canal (arrows in 4b).

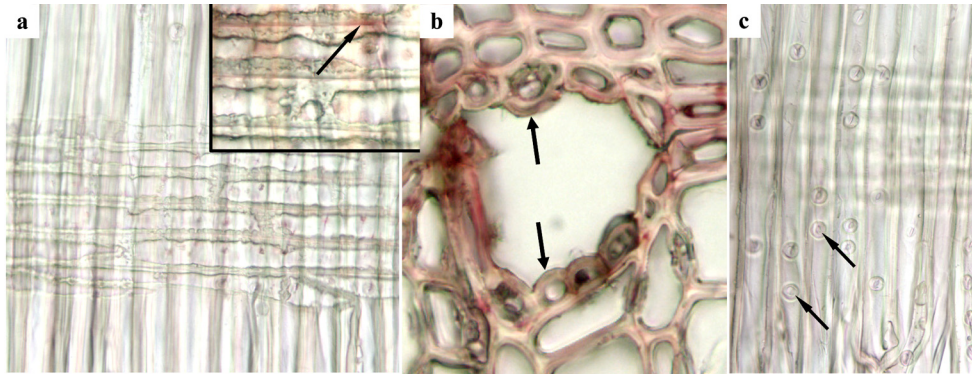


Figure 4a-c. Radial (a, c) and transverse (b) sections of RR stained treated samples: a) most rays showed no staining although some parenchyma cells did stain weakly with RR (arrow in inset) indicating irregular degradation of substrate; b) most of epithelial cells did not stain or stained pale red/pink due to pectinase effect on pectins in their walls. Note the bulging of surrounding epithelial cells (arrows) towards the empty resin canal due to swelling effect after enzyme action; c) pit-membranes (arrows) of bordered-pits in treated wood showed no staining to RR

The present results from histochemistry indicate a possible effect on the wood chips by the pectinase enzyme pre-treatment (either weak- or strong degradation of pectins). Accordingly, one can expect weakening of chips mainly due to dismantling of cell wall chemistry in the ML regions and rays before the refining phase and that this may have a positive impact on reducing energy input during refining. However, these analyses were carried out on sections removed from the outermost surfaces of wood chips where the enzyme-substrate interactions would be expected to be optimal. Therefore, the results cannot be generalized as the information on enzyme penetration inside wood chips and micro-morphological and chemical effects thereupon within chips are not known and cannot be verified from this study. In-depth analyses using immuno-electron microscopy on samples from both outside and inside of wood chips will be carried out in the future.

Conclusions

Histochemical analyses on 1 hr pectinase treated- and untreated Norway spruce wood chips showed that the enzyme pre-treatment affected the outer surfaces where pectin chemistry of cell walls was found to change/degrade. This applied to all cellular structures that are known to contain pectins in their cell walls including the compound middle lamella of fibers, ray parenchyma, epithelial cells surrounding resin canals and pit membranes of bordered-pits of fibers although the effect was found to be irregular even within a given sample. However, information on micro-morphological and chemical effects inside wood chips cannot be verified from the present study and thus the overall effects of pectinase pre-treatment on raw materials needs to be confirmed.

References

Harsveld van der Veen, J.E., Van den Ent, E. J. (1994). Selective pulping of non-wood plant species: pectin in and between fiber cell walls in plant tissue. Proc. Tappi Pulping Conference, 2: 749–757.

Hafrén, J., Daniel, G., Westermark, U. (2000). The distribution of acidic and esterified pectin in cambium, developing xylem and mature xylem of *Pinus sylvestris*. IAWA J., 21 (2): 157–168.

Naithani, S., Singh, S.V., Bhatt, O.P., Dhoundiyal, R. (2004). Alkaline peroxide mechanical pulping of *Populus deltoides*, IPPTA J., 16(2): 57-59.

Nelsson, E., Hildén, L., Sandberg, C., Fernando, D., Daniel, G. (2011). Mill scale experiences of combined sulphite pre-treatment and high intensity refining of spruce. International Mechanical Pulping Conference, X'ian, China, 182-186.

Petit-Conil, M., Hoddenbagh, J., Marc, A., Tolan, J. S. (2005). Method for mechanical pulp production. WO 2005/106110 A1.

Sterling, C. (1970). Crystal-structure of ruthenium red and stereochemistry of its pectic stain. Amer. J. Bot. 57(2): 172-175.

Collaborative Research on the Ultrastructure of Wood Fibres (CRUW)

CRUW represents a collaborative research program between the Swedish Forest Industries Eka Chemicals, Holmen, Smurfit Kappa Packaging, SCA, Stora Enso, Södra, SLU, Innventia, KTH and Mid Sweden University. The program is directed towards energy efficient processes for mechanical pulping and retention of the full fibre potential in chemical pulping. It is believed that research ideas based on insight into fibre ultrastructure can provide openings for breakthroughs in the applied area. The program forms part of the VINNOVA and Industry *"Branschforskningsprogram för skogs- och träindustrin"*.

MILJÖMÄRKT TRYCKSAK
341 051