



Effect of pectin extraction method on properties of cellulose nanofibers isolated from sugar beet pulp

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Abstract In this study, the effect of pectin extraction method on the properties of cellulose nanofibers (CNFs) isolated from sugar beet pulp (SBP) was studied. Pectin was extracted by the industrially practiced method by sulfuric acid hydrolysis or by enzymatic hydrolysis using a cellulase/xylanase enzymes mixture. The CNFs were then isolated by high-pressure homogenization and investigated in terms of their chemical composition, crystallinity, size, degree of polymerization, and re-dispersion in water after freeze-drying. The mechanical properties and surface characteristics of CNF films were also studied. The results showed that fibrillation of the de-

pectinated SBP was more efficient for the acid hydrolyzed SBP. CNFs from the acid-hydrolyzed SBP had a slightly wider diameter, higher crystallinity, viscosity, and α -cellulose content but a lower degree of polymerization than CNFs from the enzyme-hydrolyzed SBP. Owing to the presence of more residual hemicelluloses in the CNFs from the enzyme-hydrolyzed SBP, the CNFs had higher re-dispersion ability in water. CNF films from enzyme-hydrolyzed SBP displayed slightly better mechanical properties and higher water contact angle than acid-hydrolyzed CNF films.

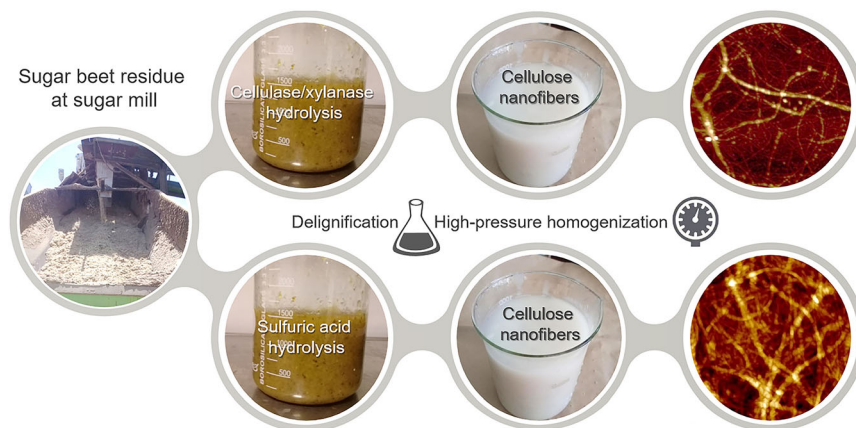
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Graphic abstract



Keywords Sugar beet pulp · Cellulose nanofibers · Acid hydrolysis · Enzymatic hydrolysis · Cellulases · Xylanases

Introduction

Sugar beet pulp (SBP) is a common agricultural residue in different areas of the world as 20% of the world production of sugar comes from sugar beets (Stevanato et al. 2019). Sugar beets are cultivated in Europe, North and South America, Asia, and a few countries in Africa where temperate climates exist. The top producer is the Russian Federation, followed by France, the United States, Germany, and Turkey. In Africa, Egypt is the leading country in sugar beet cultivation and the 8th top producer in the world. After sugar extraction, SBP residue is rich in some carbohydrate polymers and other minor components. The chemical composition of SBP is approximately 21–30% pectin, 22–25% cellulose, 22–25% hemicelluloses, 5–10% protein, lignin < 5%, phenolics < 1%, and ash < 1% (Fishman et al. 2011; Li et al. 2014).

Indeed, the cell wall structure of sugar beet is quite unique and different from those of agricultural residues such as bagasse, rice straw, etc. Most of the tissue in sugar beet is parenchymal, which is separated by vascular tissue (Dinand et al. 1999). The

parenchymal tissue is characterized by only primary wall of very thin thickness (as low as 0.1 μm). The intervascular parenchyma consists of arrays of ovoid cells having diameters ranging from 50 to 200 μm . Other parenchymal cells also exist in the vascular tissue, so called phloem parenchyma, and characterized by very thin wall with an elongated structure, with diameter of 0.4 μm and lengths of around 100 μm . Similar to the primary walls in different plants, the thin walls of the different parenchymal cells contain loosely organized cellulose microfibrils embedded in a matrix of hemicelluloses and pectin. The cellulose microfibrils are either isolated with about 3 nm diameter or organized in thin bundles with a limited number of parallel microfibrils (Dinand et al. 1999). This loose microfibrillar organization in the matrix facilitates separation of cellulose microfibrils by mechanical action.

Regarding hemicelluloses in SBP, they contain both xylan and xyloglucan polymers (Kato and Kobayashi 2000). Xylan part in SBP consists mainly of a linear (β 1 \rightarrow 4)-linked D-xylopyranosyl backbone (~ 89% of total xylan structure) with side chains at the O-2 and O-3 positions, consisting of glucose, arabinose, glucouronic acid, galactose, and rhamnose sugars. On the other hand, xyloglucan consists of (β 1 \rightarrow 4)-linked D-glucosyl units substituted at the O-6 positions with single α -D-xylopyranosyl, β -D-galactopyranosyl-(1 \rightarrow 2)- α -D-xylopyranosyl, and (α 1 \rightarrow 2)-

L-fucopyranosyl-(β 1 \rightarrow 2)-D-galactopyranosyl-(α 1 \rightarrow 2)-D-xylopyranosyl residues.

Pectin in sugar beet has rather complicated structure. It consists of different structural elements known as homogalacturonan (HG) and rhamnogalacturonan with two structures so called RG I and RG II structures. These elements are linked to each other via different suggested models (Vincken et al. 2003; Schols et al. 2009). The HG elements are linear chains of (α 1 \rightarrow 4)-linked D-galacturonic acid residues which could be acetyl-esterified at O-2 and/or O-3 and methyl-esterified at O-6 (Combo et al. 2013). The RG I elements consist of alternating rhamnose and galacturonic acid units. From the rhamnose units, linear (β 1 \rightarrow 4)-linked galactan and arabinan are branched. The branched arabinan consists of (α 1 \rightarrow 5)-linked backbones with (α 1 \rightarrow 2) and/or (α 1 \rightarrow 3)-arabino furanosyl substitutions, while RG II elements, which exist to much less extent in sugar beet than RG I, consist of galacturonic acid with side chains of different sugars (galactose, arabinose, rhamnose, and xylose). Protein and ferulic acid residues are linked to the arabinan and galactan side chains of the RG I elements (Levigne et al. 2002; Siew and Williams 2008); only very few plants have ferulic acid residues in the RG I elements, and sugar beet is one of them.

In such unique complicated cell wall structure of SBP, xylan and xyloglucan of hemicelluloses are connected to pectin through ester bonds between their hydroxyl groups and the galacturonic acid groups of pectin (Fischer et al. 1994). Cellulose microfibrils on the other hand are linked to pectin in the cell wall in similar manner to that with hemicelluloses (Zykwinska et al. 2005, 2007).

For efficient use of SBP residue, industrially valuable polymers such as pectin and cellulose should be isolated using optimized protocols. Pectin extraction from the cell wall of sugar beet requires breaking the bonds between cellulose, hemicelluloses, and pectin. Although hydrolysis using mineral acids is a common industrial method for pectin extraction, other methods, such as enzymatic hydrolysis, have also been studied (Zykwinska et al. 2008; Concha et al. 2013; Babbar et al. 2016; Pacheco et al. 2019; Abou-Elseoud et al. 2021). In addition to being a more environmentally sound approach than mineral acid hydrolysis, enzymatic hydrolysis was also found to be effective in terms of pectin yield in these previous studies. After pectin extraction, the de-pectinated SBP becomes

enriched with cellulose fibers, which can be used to isolate nanocellulose (cellulose nanofibers and nanocrystals) known to have interesting mechanical, optical, and physical properties, making them applicable in several areas (Thomas et al. 2018). The isolation of CNFs from de-pectinated SBP has been studied in past years. In these studies, the main protocol was to first remove pectin from SBP by alkali treatment, then remove lignin by bleaching, and finally isolate CNFs from the purified pulp using high-pressure homogenizers (Leitner et al. 2007; Li et al. 2014; Pinkl et al. 2017). The use of mixtures of enzymes (pectinases, hemicellulases, amylase, and endo-glucanase) to purify the cellulose fraction from residual pectin after alkali treatment and bleaching of SBP was also studied. The purified pulp was then subjected to high-pressure homogenization to produce CNFs (Holland et al. 2019; Perzon et al. 2020). In a slightly different protocol to isolate CNFs, SBP was first treated with hot nitric acid solution three times, alkali treated three times, bleached with sodium chlorite/acetic acid, treated with ultrasonication, and finally subjected to high-pressure homogenization (Agoda-Tandjawa et al. 2010). CNFs from SBP were also isolated without the removal of pectin or other polysaccharides; only a bleaching step with sodium chlorite/acetic acid was carried out, and the CNFs were isolated by high-pressure homogenization (Hietala et al. 2017); the isolated nanofibers contained significant amounts of pectin, which gave them high re-dispersion properties after drying. CNFs from SBP were also isolated without chemical treatment by direct grinding using an ultrafine grinder followed by high-pressure homogenization (Vartiainen et al. 2015). Using a different approach, SBP was subjected to steam explosion pretreatment before bleaching and ultrasonic treatment to obtain CNFs (Yang et al. 2018).

Despite successful isolation of CNFs from SBP in the above-mentioned studies, full use of the major components of SBP in industry, e.g., pectin and cellulose, is necessary for economic reasons to expand the traditional use of SBP for animal feed alone. Therefore, optimized isolation of pectin before using the cellulose fraction is mandatory. In addition, because different methods can be used for pectin extraction, the effect of these methods on the properties of isolated CNFs needs to be studied. The different methods of pectin extraction of course affect its

properties; for example, pectin isolated from sugar beet pulp using cellulase/xylanase enzymes mixture was found to have lower galacturonic acid than that produced using sulfuric acid hydrolysis but pectin isolated by the enzymes mixture was higher in neutral sugars content, degree of esterification, and molecular weight (Abou-Elseoud et al. 2021).

The aim of the current work was to study the differences in properties of CNFs isolated from SBP residue by first optimizing the pectin isolation using the conventional industrially practiced sulfuric acid hydrolysis or enzymatic treatment with a mixture of xylanase/cellulase enzymes, and then using the de-pectinated SBP to isolate the CNFs. The study also focused on the progression of fibrillation using acid- and enzymatic-hydrolyzed SBP, the re-dispersion of the dried nanofibers, and the properties of CNF films prepared by casting.

Experimental

Raw material and reagents

SBP was kindly supplied by Alnubariah Company for Sugar, Alexandria, Egypt. The chemical composition of the SBP regarding α -cellulose, pentosans, lignin, and ash content was determined according to standard methods of chemical analyses (Browning 1967); galacturonic acid content (as a measure for pectic substance) was determined according to the previously published method using *m*-hydroxybiphenyl reagent (Meseguer et al. 1998). Protein content was determined from the nitrogen content analysis by the Kjeldahl method (Sàez-Plaza et al. 2013), where protein content % = N% \times 6.25. Cellulase from *Trichoderma longibrachiatum* (β -glucosidase powder, \geq 1.0 unit/mg) and xylanase (Purified endo- β 1 \rightarrow 4)-xylanase powder, \geq 2500 units/g) from *Thermomyces lanuginosus* were purchased from Sigma Aldrich Sweden AB (Stockholm, Sweden) and used as received. Regarding activity of the enzymes, one unit of cellulase enzyme liberates 1.0 μ mole of glucose from cellulose in 1 h at pH 5.0 at 37 °C after 2 h incubation time, while one unit of xylanase liberates 1 μ mole of reducing sugar measured as xylose equivalents from xylan per min at pH 4.5 at 30 °C.

Sodium chlorite (technical grade 80%), glacial acetic acid, sulfuric acid, sodium thiosulfate,

potassium bromide, potassium bromate, hydrochloric acid, acetic acid, citric acid, and sodium hydroxide were of analytical grade. They were purchased from Fisher Scientific U.K. Ltd (Loughborough, UK) and used as received.

Extraction of pectin by enzymatic hydrolysis

Extraction of pectin from SBP using cellulase and xylanase mixture was carried out as previously published by Abou-Elseoud et al. (2021). A mixture of xylanase and cellulase in a ratio of 1:1.5 was prepared in citrate buffer at 25 °C. SBP, previously ground to pass through a 40-mesh screen, was added to the enzyme mixture at a liquor ratio of 1:15 (SBP to enzyme mixture) at 50 °C under shaking at 150 rpm for 4 h. The enzymes were then inactivated by heating at 100 °C for 5 min. The residue (de-pectinated SBP) was separated from the soluble compounds by vacuum filtration and washed with distilled water. The de-pectinated SBP yield was calculated as follows Eq. 1:

$$\begin{aligned} \text{De-pectinated SBP yield (\%)} \\ &= (\text{dry weight of SBP after extraction} / \\ &\quad \text{dry weight of SBP before extraction}) \times 100. \end{aligned} \quad (1)$$

The filtrate was centrifuged at 10,000 rpm for 10 min to remove fine particles (fines), and pectin was precipitated by the addition of ethanol at volume ratio of 3:1 ethanol to filtrate. After 2 h, the precipitated pectin was centrifuged at 10,000 rpm for 20 min, washed with 70% ethanol, centrifuged again, and dried at 40 °C for 48 h. The pectin yield was calculated as follows Eq. 2:

$$\begin{aligned} \text{Pectin yield (\%)} \\ &= (\text{dry weight of pectin/dry weight of SBP}) \times 100. \end{aligned} \quad (2)$$

Extraction of pectin by acid hydrolysis was carried out as previously published by Abou-Elseoud et al. (2021). SBP was suspended in water at a liquor ratio of 1:15 and acidified to pH 1 with sulfuric acid. It was then heated for 2 h at 85 °C under mechanical stirring. The residue was then separated from the soluble compounds by vacuum filtration and washed with distilled water.

Isolation of cellulose nanofibers

After pectin extraction, the de-pectinated SBP samples were washed with water and then treated with diluted sodium hydroxide (4% based on de-pectinated SBP weight) at 25 °C under mechanical stirring for 1 h to remove residual solubilized pectin that was not removed by washing. Then, the mixture was filtered, and the produced pulp was washed with water until reaching neutral pH. The pulp was finally bleached with a sodium chlorite/acetic acid mixture at 80 °C for 1 h (Wise et al. 1946). To isolate the CNFs, the purified pulp, at 2 wt% consistency, was mixed for 15 min in a Silverson L4RT shear mixer (Silverson Machines Ltd., Chesham, UK) followed by high-pressure homogenization using a two-chamber APV-2000 high-pressure homogenizer (SPX, Soeborg, Denmark). The pressure was maintained at 40 bar in one chamber and at 400 bar in the other chamber. The number of passes through the homogenizer varied from one to five times depending on the progression of fibrillation. The progression of the fibrillation was followed by measuring the viscosity at different passes/time through the homogenizer using a tuning-fork vibration viscometer Vibro Viscometer SV-10 (A&D Company Limited, Tokyo, Japan). The progression of fibrillation was also followed by optical microscopy using a polarizing microscope (Nikon Eclipse V100N POL, Tokyo, Japan) and the imaging software NIS-Elements D 4.30.

Characterization of isolated CNFs

The chemical composition of isolated CNFs was determined according to standard methods of analyses of α -cellulose, hemicelluloses (as pentosans), lignin, and ash content (Browning 1967). Galacturonic acid content was determined according to the previously published method using *m*-hydroxybiphenyl reagent (Meseguer et al. 1998). Protein content was determined from the nitrogen content analysis by the Kjeldahl method (Sàez-Plaza et al. 2013).

The microstructure of the SBP before and after pectin extraction was investigated using an FEI Quanta 200 scanning electron microscope (SEM; FEI Company, Eindhoven, The Netherlands). SBP samples were coated with gold prior to investigation using a sputtering coating system (Edwards Vacuum Engineering, Sussex, UK).

The microstructure of the isolated CNFs was studied using a high-resolution transmission electron microscope (TEM) (JEM-2100, JEOL, Tokyo, Japan). A drop of highly diluted CNF suspension (~ 0.02 wt%) was placed on a copper grid bearing a carbon film. After the sample was dried, phosphotungstic acid staining was applied and left to dry before the test. Staining was applied to improve contrast during imaging.

Atomic force microscopy (AFM) was also used to measure the size of the CNFs. Suspension with a concentration of 0.01 wt% was dropped onto freshly cleaved mica for scanning with a Veeco Multimode Scanning Probe (Santa Barbara, CA, USA) in tapping mode using a tip model TESPA [antimony (n)-doped Si] (Bruker, Camarillo, CA, USA). Height scans were used to measure the width in air at 22 °C using Nanoscope V software. The average values and standard deviations presented were based on 60 separate measurements. CNF suspensions (~ 2 wt%) isolated from both the enzyme- and acid-hydrolyzed SBP were freeze-dried as follows: The suspensions were stored in a freezer at a temperature of approximately -20 °C for at least 24 h prior to freeze-drying, which was conducted for 48 h using a freeze dryer (Alpha 2–4 LD Plus, CHRIST GmbH, Osterode am Harz, Germany) at a temperature of -40 °C and a vacuum of 0.12 mbar.

For the testing of re-dispersion in water, 0.1 g of the freeze-dried CNFs was dispersed in 100 mL of de-ionized water and stirred with a magnetic stirrer for 10 min at 300 rpm. Last, ultrasonic treatment was applied for 1 min using a 400 Hielscher ultrasonic processor (Hielscher Ultrasonics GmbH, Teltow, Germany). A 1-cm-diameter probe was used at an amplitude of 75%; the beaker was kept in ice water to avoid water evaporation. Light transmittance was measured immediately after ultrasonic treatment using a UV-visible spectrophotometer (Jenway 7205, Staffordshire, England) at 400–800 nm. The ultrasonic treatment was repeated at 1 min intervals, and light transmittance was measured until no noticeable change occurred. The total ultrasonic time used was 6 min for all samples. The re-dispersed nanofibers were analyzed using optical microscopy directly after re-dispersion. In addition, few drops of the methylene blue dye solution (0.1 g in 50 ml of distilled water) were added to the CNF suspensions after the ultrasonic treatment of the freeze dried nanofibers. The mixture

was left for 15 min and excess dye was removed by repeated centrifugation and washing with distilled water before imaging. The images were recorded using Optika-290 series microscope (OPTIKA, Ponteranica, Italy) for a drop of the suspension at 25 °C.

The X-ray diffraction (XRD) patterns of CNFs were recorded using an Empyrean X-ray diffractometer (PANalytical, Netherlands). The counting time was 150 s, current was 35 mA, and step size was 0.053°. The crystallinity index was calculated from the XRD patterns according to the following Eq. 3 (Sidiras et al. 1990):

$$\text{CrI} = (I_{002} - I_{\text{am}})/I_{002}, \quad (3)$$

where I_{002} is the intensity of the diffraction profile at the position of the 002 peak ($2\theta = 22.7^\circ$) and I_{am} is the intensity at approximately $2\theta = 18^\circ$. The degree of polymerization (DP) of the CNFs was determined using the bis(ethylenediamine)copper(II) hydroxide solution method. Approximately 0.1 g was used in the test (Browning 1967).

Characterization of CNF films

CNF films were prepared by casting CNF suspensions in a 9-cm-diameter Teflon petri dish. The suspensions were dried at 40 °C for 18 h in an oven with circulating air. The produced films were conditioned at 50% relative humidity for 48 h at 25 °C before testing.

The wettability of the CNF films was assessed by water contact angle measurements using an EASY-DROP measuring system, drop shape analysis control (DSA1), and evaluation software (Krüss GmbH, Hamburg, Germany). A 4 mL water drop was released onto the sample surface, and its contact angle was measured by the sessile drop technique. The reported values are the average of six measurements for each sample.

The topographical features of the CNF films were studied using the same AFM setup described above, and the root-mean-square roughness was measured from the 15 $\mu\text{m} \times 15 \mu\text{m}$ height scans. This was carried out to compare the topographical features of the surfaces of the films and their potential effect on the contact angle measurement. The corresponding amplitude (deflection) images were also collected. The reported roughness values are the average of seven

separate measurements from 15 to 15 μm height scans at different areas of the films.

The mechanical properties were measured using a Lloyd instrument (LR10 K; Lloyd Instruments, Fareham, UK) with a 1 kN load cell at 25 °C using a crosshead speed of 2 mm/min. The sample width and length were 10 and 60 mm, respectively, and the distance between the grips was 20 mm. Five specimens from each sample were measured, and the results were averaged.

Results and discussion

For effective use of the SBP residue, its pectin should be extracted first under optimized conditions. Pectin is an important food additive, and its isolation from the sugar beet should be carried out carefully to obtain the highest possible yield while maintaining its functional properties such as emulsification and gelling. After pectin extraction, the residual pulp has high cellulose content, which can be used to produce nanofibers with relatively low energy consumption. In the current work, pectin was extracted by sulfuric acid hydrolysis as it is practiced industrially or by enzymatic hydrolysis using a mixture of xylanase and cellulase enzymes; the conditions of pectin extraction by both methods were optimized in a previous publication to get highest pectin yield (Abou-Elseoud et al. 2021); the pulp residues after pectin extraction were used to isolate CNFs. In case of sulfuric acid extraction, the de-pectinated SBP residue was 43.1%, which was reached under extraction conditions of 85 °C for 2 h at pH 2. With enzymatic hydrolysis, the de-pectinated SBP residue was 61.4%, which was reached using a 1:1.5 xylanase to cellulase enzymes mixture. The lower residual de-pectinated SBP with acidic extraction could have been owing to hydrolysis of more polysaccharides into water-soluble products than was the case when using enzymes.

Figure 1 shows SBP before and after pectin extraction. As shown in Fig. 1a–c, after pectin extraction from SBP by the acid or enzymes hydrolysis, most of the parenchymal tissues between and in the vascular bundles collapsed, and thus the characteristic vascular fibers bundles became clearly visible. These vascular bundles de-folded at longer reaction times and under mechanical stirring. As Fig. 1d shows, the bundles have few microns diameter but

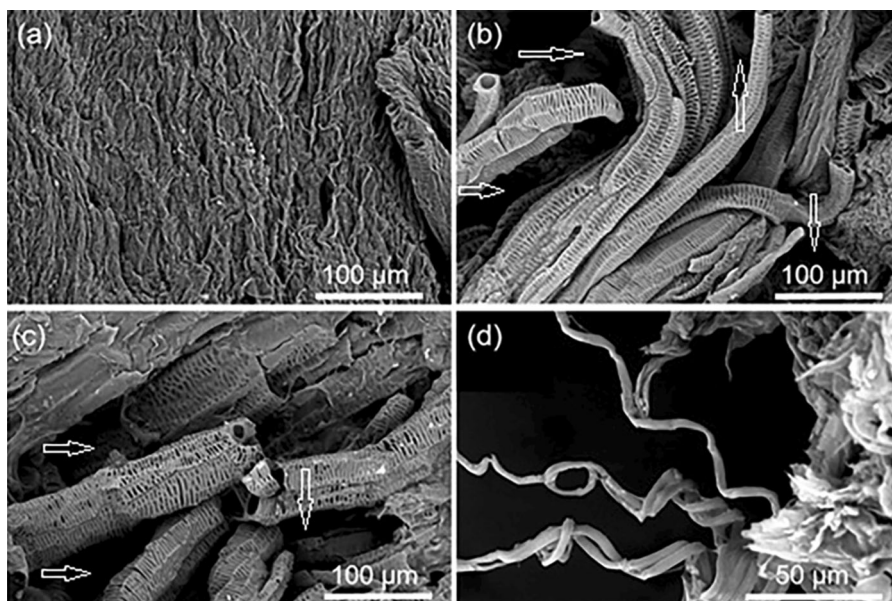


Fig. 1 SEM images of SBP **a** before pectin extraction, **b** after pectin extraction with sulfuric acid at 85 °C for 2 h, **c** after pectin extraction with a 1:1.5 xylanase/cellulase enzyme mixture, and **d** after pectin extraction with sulfuric acid at 85 °C for 4 h

hundred microns in length. The bundles contain cellulose microfibrils embedded in residual hemicelluloses and pectin. It is noted from the images that the vascular bundles obtained after pectin extraction by enzyme hydrolysis have more residual parenchymal tissue at their surfaces. Indeed, after extraction pectin from the parenchymal tissue, the remained very thin cell wall contains the cellulose microfibrils and can easily be broken by mechanical action liberating the microfibrils or microfibrils bundles (Dinand et al. 1999).

Fibrillation of CNFs

The fibrillation progression of CNFs from the depectinated SBP by acid or enzymatic hydrolysis using high-pressure homogenization was measured using viscosity measurements and optical microscopy. Previous studies have shown that increased viscosity is a good measure of the progression of fibrillation as viscosity increases with increasing fibrillation (Berglund et al. 2020; Hassan et al. 2018). This increase in viscosity is due to an increase in the surface area and the high-water binding capacity of the isolated nanofibers. As shown in Fig. 2, the viscosity of the acid hydrolyzed SBP suspension increased very rapidly during the first pass of homogenization,

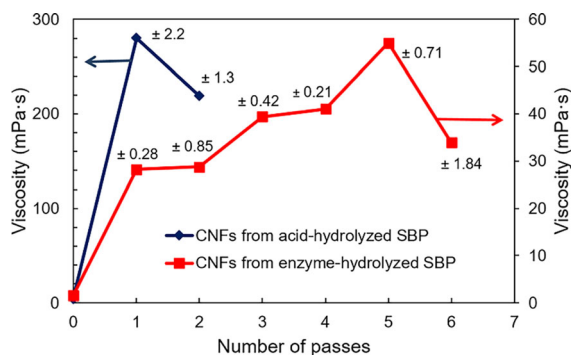


Fig. 2 Effect of number of passes through the high-pressure homogenizer on viscosity of isolated nanofibers

followed by a decrease; the maximum viscosity was 280 mPa·s after the first pass. The temperature increased from 25.5 to 43 °C after two passes, which could also cause a decrease in the measured viscosity. The viscosity of the enzyme hydrolyzed SBP residue increased to a much less extent and reached a maximum after five passes; the maximum viscosity was 55 mPa·s and the temperature reached 43 °C. These results indicate faster isolation of CNFs when using the acid hydrolyzed SBP residue.

These results were also confirmed by optical microscopy images taken during the high-pressure homogenization. These images are shown in Figs. 3

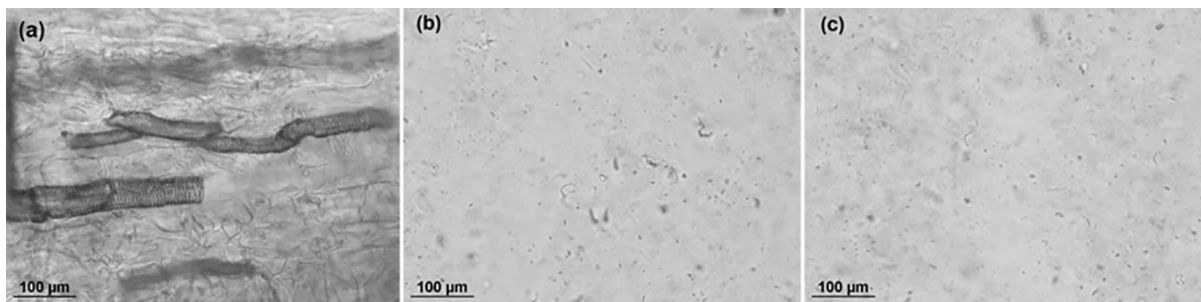


Fig. 3 Optical microscopy images of acid-hydrolyzed SBP after passage through the high-pressure homogenizer **a** zero, **b** one, and **c** two times

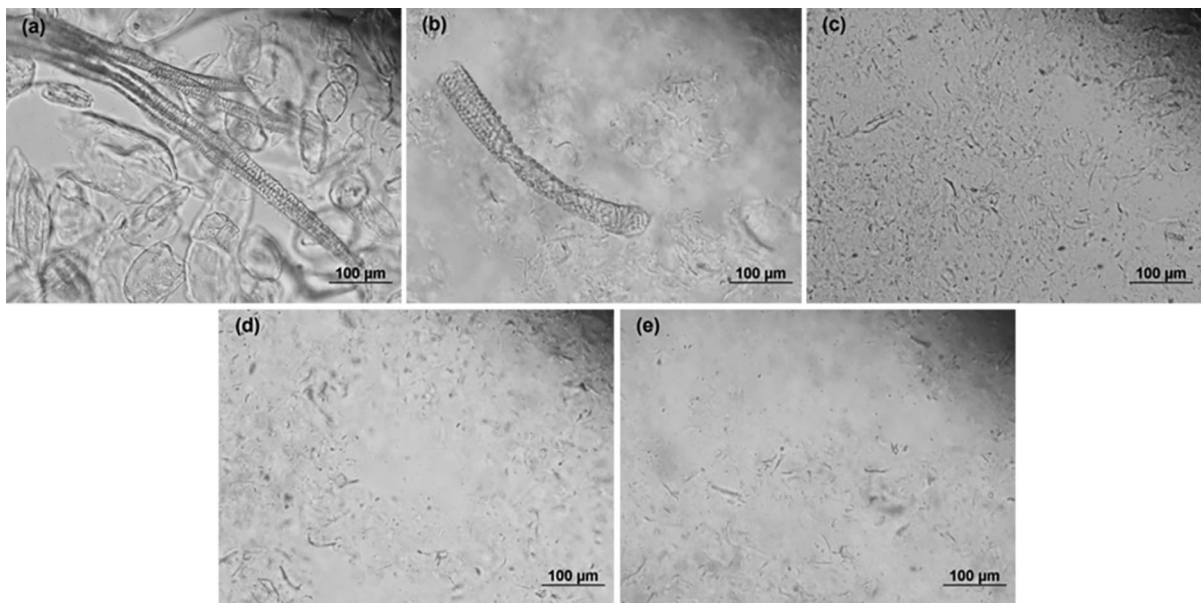


Fig. 4 Optical microscopy images of enzyme-hydrolyzed SBP after passage through high-pressure homogenizer **a** zero, **b** one, **c** three, **d** five, and **e** six times

and 4 for the acid-hydrolyzed and enzyme-hydrolyzed SBP, respectively. The micrographs show faster disappearance of the micro-sized fibers bundles and also the non-fibrous very thin-walled flatten parenchyma cells in the ground of the image (Fig. 3a) of the acid-hydrolyzed SBP than with the enzymatic hydrolysis (Fig. 4a). Upon high-pressure homogenization, the nonfibrous parenchymal cells disappeared, as well as the micro-sized xylem, which disintegrated into nano-sized fibrils and became non-visible under the magnification of the optical microscope.

The difference in the progression of fibrillation and viscosity values could be interpreted from chemical analyses of SBP and the nanofibers as seen in Table 1.

SBP contains mixture of different polymers mainly pectin, cellulose, and hemicelluloses exist in very complex structure and connected together via different kinds of bonds. Hemicelluloses in SBP consist mainly of xylan (~ 89% of which are xylose backbone) and xyloglucan polymers (Kato and Kobayashi 2000) as mentioned in the Introduction. These hemicelluloses are chemically linked to pectin through ester bonds with pectin (Fischer et al. 1994). Cellulose microfibrils in SBP are linked to pectin in the cell wall in similar way to that of hemicelluloses (Zykwinska et al. 2005, 2007). Evidence also exists regarding linkages between pectin, hemicelluloses, and cellulose via hydrogen and chemical bonding (Fry 1986; Iiyama

Table 1 Chemical composition of sugar beet pulp (SBP) and the isolated CNFs

Materials	α -Cellulose (%)	Pentosans (%)	Galacturonic acid (%)	Lignin (%)	Protein content (%)	Ash (%)
SBP	38.02 \pm 2.24	18.20 \pm 2.12	19.40 \pm 1.27	3.85 \pm 0.21	10.13 \pm 0.93	2.77 \pm 0.42
CNF acid hydrolysis	87.80 \pm 3.60	6.96 \pm 0.79	2.44 \pm 0.47	0.45 \pm 0.16	0.31 \pm 0.01	1.10 \pm 0.08
CNF enzymes hydrolysis	76.90 \pm 1.60	17.09 \pm 1.39	2.21 \pm 0.19	0.52 \pm 0.5	0.25 \pm 0.02	1.21 \pm 0.05

et al. 1994). Therefore, extraction any of these components depends on cleaving the links between these polysaccharides. In addition to the aforementioned polymers in SBP, considerable amount of protein exists in SBP which is linked to pectin (Levigne et al. 2002; Siew and Williams 2008).

As shown in Table 1, after pectin extraction by acid or enzymatic hydrolysis followed by bleaching of SBP, higher α -cellulose content (\sim 88%) was found with the acid-hydrolyzed SBP than with the enzyme-hydrolyzed SBP (\sim 77%). This means that with the acid hydrolyzed SBP, removal of most of the hemicelluloses and pectin, which are chemically linked to cellulose fibers (Fry 1986; Iiyama et al. 1994) took place resulting in easier fibrillation and much faster increase in viscosity. The hemicelluloses content (estimated as pentosans) was \sim 7.0% and 17% for CNFs from the acid- and enzyme-hydrolyzed SBP, respectively. In fact, majority of hemicelluloses (xylans and xyloglucan) in sugar beet pulp are pentoses as previous studies proved (Kato and Kobayashi 2000). That's why pentosans were taken as indication for hemicelluloses. Other hexoses also exist in xylan and xyloglucan structures such as glucose (which also the cellulose monomer) and galactose. It is important to mention that pectic substance in SBP also contains pentose sugars (arabinose). Residual pectic substance in the nanofibers, estimated as galacturonic acid, was 2.44% and 2.21% in case of acid- and enzyme-hydrolyzed CNFs, respectively, compared to \sim 19% in the SBP. It should be also noted that pectic substance in SBP consists of galacturonic backbone, in addition to branches of galactan and arabinan from the rhamnagalacturonan units (Schols et al. 2009).

The higher viscosity at the end of fibrillation for the acid-hydrolyzed SBP than for the enzyme-hydrolyzed SBP could be attributed to the higher cellulose content of the former, i.e., more nanofibers were liberated in

the water suspension by high-pressure homogenization. In fact, hemicelluloses and pectin in SBP have low molecular weight and viscosity, and thus their presence in the isolated nanofibers is not expected to considerably affect the viscosity of the CNF suspension. The presence of residual pectin with nanofibers isolated from SBP was reported in a previous publication, despite the strong alkali extraction used in these previous studies (Perzon et al. 2020). Owing to the bleaching of SBP, traces of lignin were detected in both types of nanofibers (0.5%). The DP of cellulose was 639 and 894 for nanofibers isolated from the acid- and enzyme-hydrolyzed SBP, respectively. The lower DP of the nanofibers from the acid hydrolyzed SBP was more likely owing to more degradation by the action of the acid during pectin extraction than that in case of using the enzymes. It should be pointed out here that cellulases can definitely cause degradation of cellulose but the extent of their effect depends on the conditions used in the treatment, especially their dose and also length of duration. The conditions used in the current work was based on our previous study on using the cellulase/xylanase mixture for optimizing pectin extraction where the minimum possible amount that could give good yield of pectin was used (Abou-Elseoud et al. 2021).

Microstructure of isolated CNFs

Regarding the dimensions of the isolated nanofibers, TEM images showed that the diameters of the nanofibers isolated from both types of SBP residue were very homogenous. The diameters were 6–10 nm and 3–5 nm for nanofibers isolated from acid- and enzyme-hydrolyzed SBP, respectively (Fig. 5a and b, respectively). This means that elementary cellulose fibrils could be easily isolated from both types of SBP residue using high-pressure homogenization after one

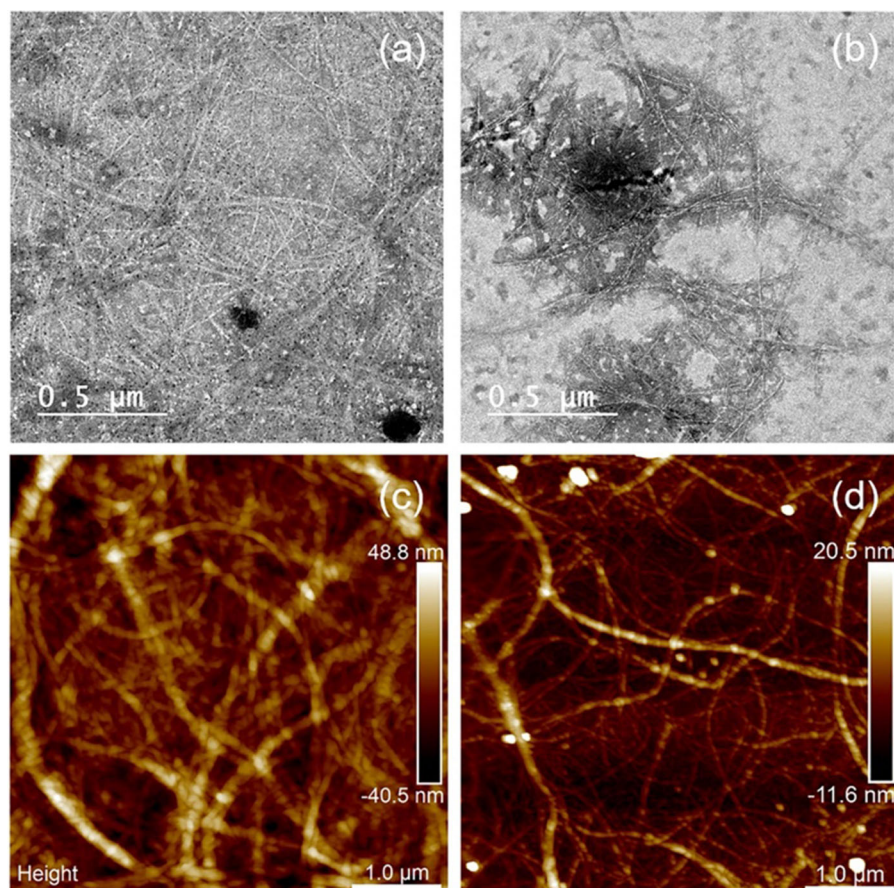


Fig. 5 TEM images of CNFs isolated from **a** acid-hydrolyzed SBP and **b** enzyme-hydrolyzed SBP. AFM images in height mode of CNFs isolated from **c** acid-hydrolyzed SBP and **d** enzyme-hydrolyzed SBP

and five passes through the homogenizer for acid- and enzyme-hydrolyzed SBP, respectively. The length of the nanofibers was several microns for both types of SBP residue. AFM images confirmed the trend of the diameter of isolated nanofibers for both types of SBP residue; the measured height were 10 ± 5 nm and 7 ± 4 nm for the acid- and enzyme-hydrolyzed SBP, respectively (Fig. 5c and d, respectively). The size distribution of isolated CNFs measured from AFM height scans is shown in Supplementary Information Figure S1.

A comparison of the diameter of CNFs in the current work to that in other publications is shown in Table 2. The diameter of CNFs obtained in the current work was among the smallest, and more interestingly, that for the acid hydrolyzed SBP was reached after only one pass through the high-pressure homogenizer. Other studies where similar widths of CNFs were

isolated from SBP required many passes through high-pressure homogenizers (Agoda-Tandjawa et al. 2012; Pinkl et al. 2017) or the use of many enzymes plus many passes through the homogenizers (Perzon et al. 2020).

Crystallinity

The XRD patterns of CNFs isolated from acid- and enzyme-hydrolyzed SBP residues are shown in Fig. 6. Both types of CNFs have a cellulose I structure, namely, peaks at $\sim 22^\circ$ and 16° , which correspond to diffraction from the (200) and (110) planes, respectively. The calculated degree of crystallinity was higher for CNFs isolated from acid-hydrolyzed SBP ($\sim 74\%$) compared to that for CNFs isolated from enzyme-hydrolyzed SBP ($\sim 71\%$). The higher crystallinity of the acid-hydrolyzed CNFs means that the

Table 2 Comparison between sizes of CNFs isolated from SBP residue in the current work and in previous publications under the conditions indicated

Pectin extraction	Purification process	Fibrillation	Size (nm)	References
Sulfuric acid pH 1, 85 °C, 2 h	Dilute alkali, 25 °C, bleaching	High-pressure homogenizer 1 pass	6–10	Current work
Xylanase/cellulase enzymes pH 5, 50 °C, 4 h	Dilute alkali, 25 °C bleaching	High-pressure homogenizer 5 pass	3–5	Current work
Alkali treatment 80 °C, 2 h	Bleaching	Microfluidizer 11 pass	20–40	Perzon et al. (2020)
Mixture of enzymes	Bleaching	Microfluidizer 11 pass	~ 5	–
Alkali treatment 80 °C, 2 h & mixture of enzymes	Bleaching	Microfluidizer 11 pass	20–40	–
Homogenization, pH 9, 2 h & mixture of six enzymes 40 °C for 24 h	None	Microfluidizer 18 min	5	Holland et al. (2019)
None	Bleaching	Steam explosion/ultrasonic treatment	10–50	Yang et al. (2018)
Alkali treatment 82 °C, 2 h	Bleaching	Microfluidizer	≤ 100	Hietala et al. (2017)
None	Bleaching	5 passes	≤ 100	
Alkali treatment, 80 °C, 2 h	Bleaching	High-pressure homogenizer 20 passes	5–10	Pinkl et al. (2017)
Alkali treatment, 80 °C, 2 h	Bleaching	High-pressure homogenizer 10 passes	10–70	Li et al. (2014)
Nitric acid, 0.1 N, 85 °C, 30 min, repeated 3 × Alkali 80 °C, 0.5 h, repeated 3 ×	None	Ultrasonication & high-pressure homogenizer 65 °C and 10 passes	2–15	Agoda-Tandjawa et al. (2010)
Alkali treatment 80 °C, 2 h	Bleaching	High-pressure homogenizer 10–15 passes	30–100	Leitner et al. (2007)
Alkali treatment, 80 °C, 2 h	Bleaching	High-pressure homogenizer, 65 °C 15 passes	≤ 20*	Dinand et al. (1999)

* Estimated from the scale bar on the TEM image since the actual diameter was not mentioned

acid method was more effective in dissolving the amorphous parts of cellulose and hemicelluloses during pectin extraction. This is in accordance with the chemical analysis results mentioned previously. Another peak of low intensity at $2\theta = 8.5\text{--}9.0^\circ$

appeared in the pattern, which could be assigned to residual pectin (Wathoni et al. 2019); the band was sharper and less broad in case of CNFs isolated from acid-hydrolyzed SBP than that of enzyme-hydrolyzed

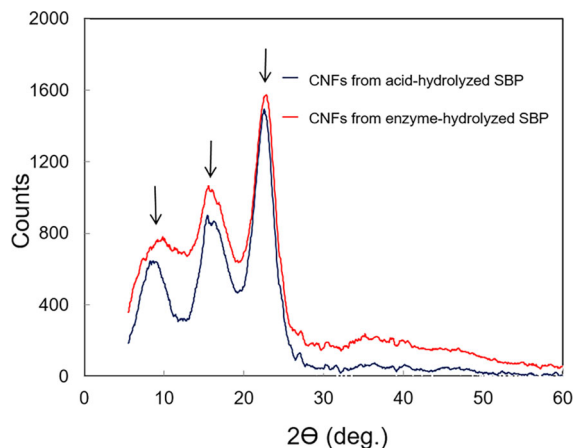


Fig. 6 XRD patterns of CNFs isolated from acid- and enzyme-hydrolyzed SBP

one, which may indicate different crystallinity of the pectic residues.

Redispersion of freeze-dried CNFs

The redispersion of dried CNFs is a challenge because of the strong aggregation of the nanofibers by extensive hydrogen bonding upon drying. Previous studies on nanofibers isolated from untreated SBP (without pectin removal) showed the possibility of redispersing the dried nanofibers in water into individual nanofibrils up to 80% of the dried nanofibers (Hietala et al. 2017). This was attributed to the presence of residual pectin and hemicelluloses attached to the isolated nanofibers. In the current study, redispersion of freeze-dried CNFs isolated from acid- and enzyme-hydrolyzed SBP was tested using light transmittance measurements (Kangas et al. 2014). Cellulosic nanofibers isolated by chemical-mechanical treatment as in the current work form colloidal suspensions with good transparency because of their nano-dimensions, whereas aggregates of microfiber size are opaque and white. Theoretically, the finer the nanofibers, the higher the transparency of the suspension owing to the higher light transmittance. As shown in Fig. 7, the non-dried CNFs from the acid- and enzyme-hydrolyzed SBP had comparable light transmittance owing to their similar diameters, as shown above in the AFM and TEM images. Upon freeze-drying and redispersion in water, the CNFs isolated from the enzyme-hydrolyzed SBP showed much higher light transmittance than the CNFs isolated from the acid-

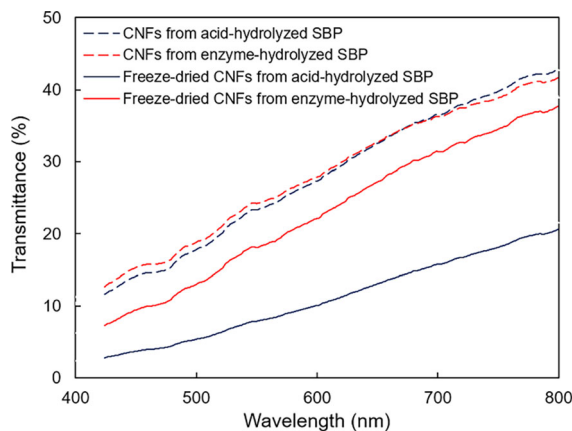


Fig. 7 Light transmittance of CNF aqueous suspensions before and after freeze-drying and redispersion in water

hydrolyzed SBP, i.e., the CNFs isolated from the enzyme-hydrolyzed SBP showed greater redispersion. This could be attributed to the higher hemicelluloses content and pectin residues in CNFs isolated from the enzyme hydrolyzed SBP.

The presence of these non-cellulosic residues hinders aggregation of the nanofibers upon drying. At 800 nm (the highest light transmittance obtained), freeze-dried CNFs from enzyme-hydrolyzed SBP retained 90% light transmittance after redispersion in water as compared to the non-dried CNFs, whereas freeze-dried CNFs isolated from acid-hydrolyzed SBP retained only 51% light transmittance. The presence of aggregates after redispersion of the CNFs was clearly seen in the optical microscopy images, which show many more aggregates for the redispersed CNFs isolated from acid hydrolyzed SBP (Fig. 8 and Figure S2).

CNF films

Films made by casting CNF suspensions were prepared and their surface properties were characterized using water contact angle measurements and AFM. Their mechanical properties were also investigated.

Topography and wettability of CNF films

Figure 9 shows the captured AFM scans, where the root-mean-square roughness was measured from the height images to evaluate and compare any differences of the topographical features. Height and

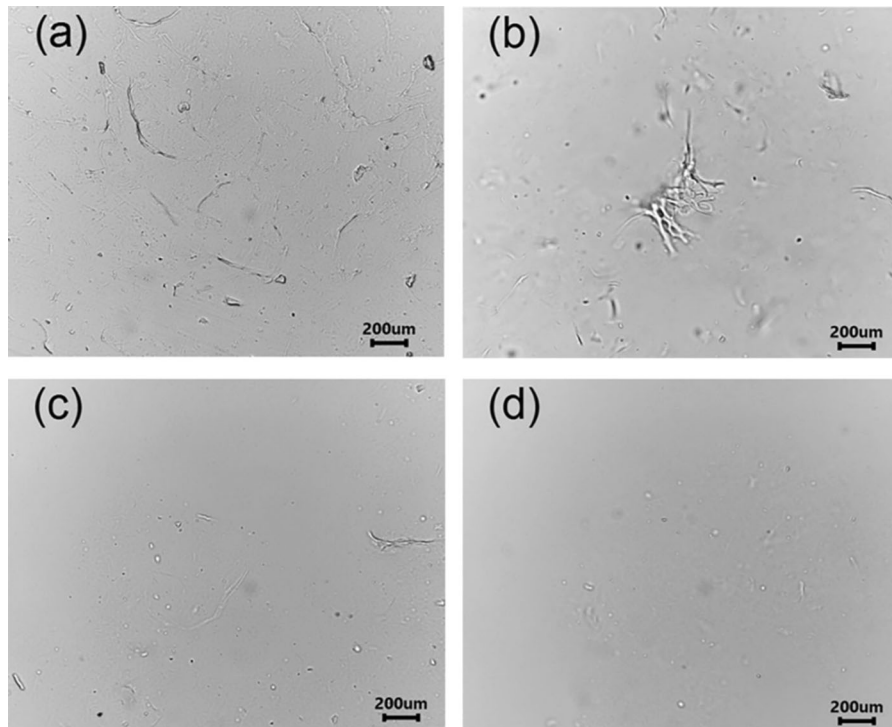


Fig. 8 Optical microscopy images of redispersed CNFs isolated from **a, b** acid-hydrolyzed SBP and **c, d** enzyme-hydrolyzed SBP at two different magnifications

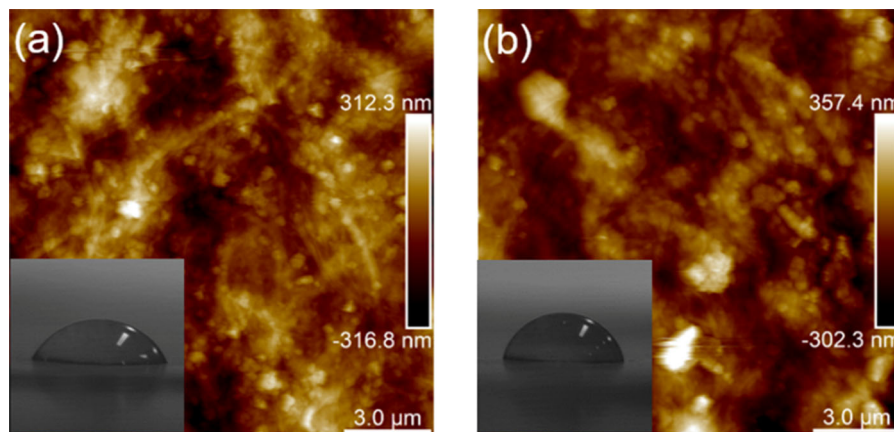


Fig. 9 AFM height image images of CNF films from **a** acid-hydrolyzed SBP and **b** enzyme-hydrolyzed SBP. Insets: Photographs of the respective water contact angles

corresponding amplitude AFM images are provided in Supplementary Information Figure S3. Water contact angles are also displayed to compare the wettability behavior of the CNF films from acid- and enzyme-hydrolyzed SBP.

The CNF films displayed similar surface characteristics, with intertwined yet visually distinguishable

nanofibers and spherical particles, as shown in Fig. 9a, b. Their surface roughness, measured from the height scans, were 81 ± 6 nm and 76 ± 6 nm for CNFs isolated from acid- and enzyme-hydrolyzed SBP, respectively. CNF films isolated from acid-hydrolyzed SBP displayed slightly higher wettability, with a water contact angle of $56^\circ \pm 1.9^\circ$, whereas that of enzyme-

hydrolyzed SBP was $61^\circ \pm 1.3^\circ$ (Fig. 9a, b, insets). It is well known that the water contact angle correlates with the topographical structure of the surface (Herrera et al. 2014). In addition, the wettability of cellulosic surfaces toward water has previously been reported to decrease upon the reduction of hemicelluloses for wood (Hosseinaei et al. 2011) and with the reduction of hemicelluloses and pectin for CNFs from carrot residue (Berglund et al. 2020). In the current work, although CNFs isolated from enzyme-hydrolyzed SBP had more hemicelluloses than those isolated from acid-hydrolyzed SBP, films from the latter had a slightly higher water contact angle. This could be owing to the higher roughness of the surface of the CNF films isolated from the acid hydrolyzed SBP.

Mechanical properties CNF films

The mechanical properties (maximum tensile strength, Young's modulus, and strain at maximum load) of CNF films prepared from both types of isolated nanofibers were measured. Stress–strain curves for representative samples are shown in Supplementary Information Figure S4. CNF films isolated from acid- and enzyme-hydrolyzed SBP had close tensile strength values (49 ± 5 and 52 ± 6 MPa, respectively). Young's modulus was 2.2 ± 0.6 and 2.8 ± 0.7 GPa and strain was $6.7 \pm 0.8\%$ and $4.3 \pm 0.6\%$, for nanofibers isolated from acid- and enzyme-hydrolyzed SBP, respectively. This higher Young's modulus could be owing to the higher DP of nanofibers from the enzyme hydrolyzed SBP. The DP values for CNFs isolated from enzyme- and acid-hydrolyzed SBP were 639 and 894, respectively. The relatively lower mechanical properties of CNF films in the current study as compared to those reported in previous studies could be attributed to the following reasons. In the current study, acid hydrolysis (pH adjusted to 1) and cellulases enzymes were used in the extraction of pectin. This have resulted in greater degradation of the cellulose chains than in other studies where alkali treatment was used to remove pectin (Leitner et al. 2007; Hietala et al. 2017; Pinkl et al. 2017; Perzon et al. 2020). Alkali treatment conditions used in the previous studies removed almost all pectin, hemicelluloses, and short-chain cellulose, and thus there was a lower possibility of degrading cellulose than when using acid or cellulase

enzymes. In addition, in one of these studies (Hietala et al. 2017), the films from the isolated nanofibers were prepared by hot pressing at elevated temperatures (110°C and 11 MPa pressure for 30 min), whereas those prepared in the current work were dried without pressure.

Conclusions

This study has shown that the removal of pectin using sulfuric acid- or xylanase/cellulase enzymatic hydrolysis leads to a residue with a high cellulose content and thus to a suitable raw material for production of CNFs. The progression of CNF isolation from de-pectinated SBP and the width of the isolated CNFs was dependent on the method of extracting pectin. CNFs with homogenous widths similar to those of elementary cellulose fibrils (approximately 5–10 nm) from SBP de-pectinated using sulfuric hydrolysis were easily isolated after one pass through a high-pressure homogenizer, whereas five passes were needed for enzyme de-pectinated SBP. Under the conditions used in this study, the extraction of pectin with sulfuric acid removed most of the hemicelluloses and pectin, resulting in easier fibrillation of cellulose fibers than was the case when extracting pectin with enzymes. However, the presence of more residual hemicelluloses for CNFs isolated from enzyme-hydrolyzed SBP resulted in higher redispersion of the freeze-dried nanofibers for CNFs isolated from acid-hydrolyzed SBP. Because of the greater degradation of cellulose during pectin extraction for the acid-hydrolyzed SBP, the prepared films had a lower tensile modulus than those for the enzyme-hydrolyzed SBP. Finally, comparing the mechanical properties of CNF films in the current study to those previously published ones, where pectin was removed mainly by alkali treatment, the extraction of pectin from SBP by industrially practiced mineral acid hydrolysis or by using cellulase enzymes resulted in nanofibers with lower mechanical properties than in the case of using alkali treatment in pectin extraction.

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Availability of materials and data All data generated or analyzed during this study are included in this published article (and its Supplementary Information files).

Declarations

Conflict of interest The authors declare no competing interests.

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