# Preparation of Polyuronic Acid by *Acetobacter xylinum*

Jin Zhao, Hao Ren,* Dongliang Xu, and Shigetoshi Omori

Water-soluble polyuronic acid was prepared *via Acetobacter xylinum* in this study. The structural features of the two polyuronic acid samples were characterized by gel permeation chromatography, Fourier transform infrared (FTIR) spectroscopy, and proton nuclear magnetic resonance ($^1$H-NMR) spectroscopy. It was found that when using glucuronic or galacturonic acid as a substrate, with samples incubated at 20 °C to 30 °C in the fermentation medium for 3 weeks to 6 weeks, water-soluble polyuronic acid products could be collected from the colloids that formed on the surface of the medium. The yield of polyuronic acid was approximately 25 wt.%. The weight-average molecular weight of the product was 150,000 to 390,000, and the polydispersity was approximately 1.01 to 1.35. These results indicated that polyuronic acid can be easily prepared by a polymerization method using *Acetobacter xylinum*. The product polymers possessed high water solubility. The FTIR and $^1$H-NMR spectra indicated glycosidic linkages were successfully formed.

*Keywords: Polyuronic acid; Glucuronic acid; Galacturonic acid; Acetobacter xylinum*

*Contact information: Jiangsu Co-Innovation Center for Efficient Processing and Utilization of Forest Resources, Jiangsu Provincial Key Lab of Pulp and Paper Science and Technology, Nanjing Forestry University, Nanjing, Jiangsu Province, 210037, China;*Corresponding author: renhao@njfu.edu.cn*

## INTRODUCTION

Polysaccharides are macromolecules possessing structural and functional diversity. They are polymeric carbohydrate molecules composed of long chains of monosaccharide units bound together by glycosidic linkages, and on hydrolysis they give the constituent monosaccharides or oligosaccharides. They range in structure from linear to highly branched. Examples include storage polysaccharides such as starch and glycogen, and structural polysaccharides such as cellulose and chitin. Polysaccharides are the most popular polymeric materials to prepare nanoparticles for drug delivery. Due to the presence of numerous derivable groups on molecular chains, they can be easily modified chemically and biochemically (Jacob *et al.* 2018). They are also widely used in diet food and moisturizing cosmetics.

In general, natural polyuronic acids can only be acquired after regioselective oxidation of natural glucans, such as cellulose. Cellulose is the major polymeric component of lignocellulosics. This β-(1,4)-linked glucan is not only the most abundant organic compound, but also the most intensively used raw material in the production of paper, panel products, chemicals, and other industrial compounds, such as various esters and ethers (Elboutachfaiti *et al.* 2011). Recently, oligoglucuronans had been reported to be produced by using a monolithic enzymatic microreactor (Tavernier *et al*. 2008). Different forms of polyglucuronic acids such as those of low molecular weight obtained after enzymatic degradation of β-(1,4)-polyglucuronic acids have been tested for biological activities with success (Elboutachfaiti *et al.* 2011). A xanthaouronic acid sodium salt called xanthouronan

was produced from xanthan by regioselective oxidation with NaOH/NaBr using TEMPO as catalyst (Delattre *et al*. 2015). Cellulose derivatives are widely used in various fields. Cellulose itself is insoluble in most solvents, and only substitution reactions of hydroxyl groups with carboxymethyl ether or sulfate ether groups result in soluble cellulose compounds. The oxidation of primary hydroxyl groups of polysaccharides to carboxylate groups could also increase the water solubility. It is difficult to obtain polyuronic acid with high water solubility and high molecular weight.

Twenty years ago, regioselective oxidation of $C_6$ primary hydroxyls using 2,2,6,6-tetramethylpiperidine-1-oxyl radical (TEMPO) was developed, and a water-soluble β-(1,4)-D-polyglucuronic acid, named cellouronic acid, can be prepared from regenerated cellulose (Isogai and Kato1998). During the same period, several natural sources of water-soluble β-(1,4)-D-polyglucuronic acids have been identified, all of which were from microorganisms (Heyraud *et al*. 1993; Ray *et al*.1995a,b, 2006). But the amount of the polyglucuronic acid obtained from microorganisms was too limited to satisfy the industrial demand.

A method to prepare polyuronic acids by biological polymerization was investigated in this study. Glucuronic and galacturonic acids were incubated with *Acetobacter xylinum* using almost the same medium as used by Hestrin and Schramm (1954). The results indicated that polyuronic acids with a high degree of polymerization and high water solubility can be synthesized using D-glucuronic and D-galacturonic acids as monomers. This method is more environmentally friendly than TEMPO oxidation (Isogai and Kato1998).

## EXPERIMENTAL

### Materials

D-glucuronic acid (GC ≥ 98%) and D-(+)-galacturonic acid monohydrate (≥ 97%) were purchased from Sigma-Aldrich (Oakville, Canada). *Acetobacter xylinum* (ATCC# 53524) was purchased from GAIDE Chem (Nanping, China). Peptone, yeast extract, sodium phosphate dibasic anhydrous ($Na_2HPO_4$), and citric acid monohydrate were all AR reagent grade and were purchased from Aladdin (Shanghai, China). Deuterium oxide (D% = 99.9%, Purity=> 99.99%) was purchased from Beijing SeaSkyBio Technology Co. Ltd. (Beijing, China). All of the reagents were used as received without further purification.

### Methods

*Preparation of the polyuronic acid*

Glucuronic and galacturonic acids were incubated with *A. xylinum* using almost the same conditions used by Hestrin and Schramm (1954). The detailed incubation process was as follows. Five grams of peptone, 5 g of yeast extract, 2.7 g of $Na_2HPO_4$, and 1.15 g of citric acid monohydrate were dissolved in 1 L of deionized water. The solution was divided into four 500-mL Erlenmeyer flasks (each of which contained approximately 250 mL of the solution). The flasks were sterilized in an autoclave at 121 °C for 15 min. After being cooled down, the flasks were kept in a refrigerator as a stock solution. When they were used for preparing polyuronic acid, 2.5 g of glucuronic or galacturonic acid were added into each flask. The pH was then adjusted to 5.0 with 1% sodium hydroxide solution and sterilized in an autoclave at 121 °C for half a minute (The power of autoclave was turned off at the moment the temperature reached 121 °C). It was important not to hold the

maximum temperature for long because incubation would have changed the sample status during sterilization. After cooling down, *A. xylinum* was added and the solution was maintained at 30 °C. The bacterium was allowed to grow for 3 to 4 weeks, like a gel in the medium. Water-soluble polyuronic acid products were collected from the colloids that were floating on the surface of the medium after 6 weeks. The collected gels were washed twice by deionized water or three times by 7% $NaClO_2$ solution, products were obtained by drying at 40 ºC vacuum drying oven after being filtered by a Buchner funnel.

*Molecular weight of the polyuronic acid*

Gel permeation chromatography (GPC) analysis was performed on the polyuronic acid samples using a Waters Alliance E2695 GPC system (Varian Inc., Palo Alto, CA, USA), equipped with a Waters 2998PDA detector. An Ultrahydrogel 500 water soluble gel column (Waters) was employed and deionized water was used as the eluent (flow rate = 0.8 mL/min). Calibration for the weight-average molecular weight ($M_w$), number-average molecular weight ($M_n$), and polydispersity ($D$) was performed using series standard dextran ($M_w$= 401000, 277000, 43500, 20600, 12600, 9600, and 4400, all units above are g/mol).

*FTIR analysis of the polyuronic acid*

The chemical structure of polyuronic acid samples was evaluated by Fourier transform infrared (FTIR) spectroscopy using a Tensor 27 FTIR spectrophotometer (Bruker, Saarbrücken, Germany). The spectra were recorded from 400 $cm^{-1}$ to 4000 $cm^{-1}$ with a resolution of 4 $cm^{-1}$ and 32 scans using a KBr disc containing 3% of the finely ground samples.

*$^1$H-NMR spectroscopy*

The hydrogen nuclear magnetic resonance ($^1$H-NMR) spectra of the polyuronic acid samples were recorded at 25 °C using an AVANCE III 600 MHz instrument (Bruker) equipped with a cryogenically cooled 5 mm TCI gradient probe with inverse geometry. The prepared polyuronic acid (30 mg) was dissolved in $D_2O$ (0.55 mL) for the NMR analysis.

*X-ray diffraction of the polyuronic acid*

The X-ray diffraction (XRD) pattern of the polyuronic acid was recorded on an Ultima IV (Rigaku,Tokyo, Japan) with a Ni-filter and Cu-Kα radiation (30 kV and 40 mA) using the diffraction method.

## RESULTS AND DISCUSSION

### Yields of the Polyuronic Acids

The yields of the two polyuronic acids were calculated and are shown in Table 1. Under the same planting conditions, either polyglucuronic or polygalacturonic acid was harvested with a yield of approximately 25 wt.% with respect to the starting materials. This method is simpler and more economic compared with the previous method based on cellulose oxidation (Isogai and Kato1998).

**Table 1.** Yields of the Polyuronic Acids

| Sample | Raw Material (g) | Product (g) | Yield (%) |
|---|---|---|---|
| D-Glucuronic Acid | 2.52± 0.02 | 0.61± 0.03 | 24.2± 1.2 |
| D-Galacturonic Acid | 2.49± 0.02 | 0.59± 0.02 | 23.7± 1.0 |

## Molecular Weights of the Polyuronic Acids

The molecular weights of the polyglucuronic and polygalacturonic acids are shown in Table 2. It was apparent that both the $M_w$ and $D$ ($M_w/M_n$) of the two polyuronic acids were similar. The $M_w$ values of the two polyglucuronic acids reached 150000 to 390000, and the $D$ values ranged from 1.01 to 1.35. These results indicated that under similar biochemical conditions for preparing bacterial cellulose, biological polymerization occurred largely in between the monomeric uronic acids studied. Because the products have a high water solubility, the polymers showed higher $M_w$ values and lower $D$ ratios when washed with water than with the 7% $NaClO_2$ solution. This data indicated that small-sized molecules might be lost during the purification process.

**Table 2.** Molecular Weight of the Polyuronic Acids

| Sample | Purification Reagent | $M_w$ (g/mol) | $M_n$ (g/mol) | $D$ ($M_w/M_n$) |
|---|---|---|---|---|
| Poly-glucuronic Acid | $H_2O$ | 396708 | 392305 | 1.01 |
| | 7% $NaClO_2$ | 164357 | 125864 | 1.31 |
| Poly-galacturonic Acid | $H_2O$ | 163540 | 161286 | 1.01 |
| | 7% $NaClO_2$ | 151257 | 147338 | 1.35 |

## FTIR Spectra of the Polyuronic Acid

The FTIR spectra of the polyglucuronic and polygalacturonic acids are shown in Fig. 1.

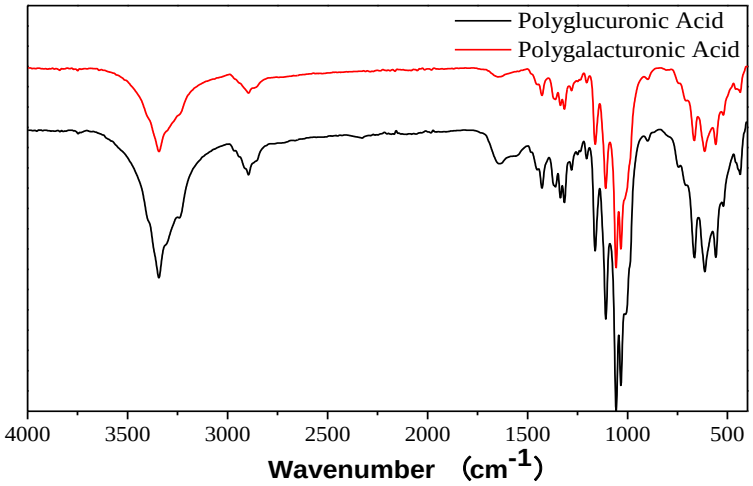


**Fig. 1.** FTIR spectra of the polyuronic acids

Intense signals were observed at 1377 $cm^{-1}$, 1311 $cm^{-1}$, and 1235 $cm^{-1}$ in both spectra, which corresponded to unsymmetrical ether linkages of β-1,4-glycosidic bonds. Additionally, the absorption band within the range 3200 $cm^{-1}$ to 3400 $cm^{-1}$ corresponded to

intermolecular and intramolecular hydrogen bonds. The band at 2923 $cm^{-1}$ was attributed to the O-H stretching vibration of the –COOH group, and the bands at 1644 $cm^{-1}$ and 1547 $cm^{-1}$ corresponded to the stretching vibration of aldehyde and carboxylate ions. These observations confirmed that D-glucuronic acid (GC ≥ 98%) and D-(+)-galacturonic acid monomers were successfully connected by biological polymerization.

## $^1$H-NMR and XRD Analysis

The $^1$H-NMR (Fig. 2) analysis indicated signals in the 2 ppm region for *O*-acetyl groups. The main group of signals in the region between 3 ppm to 4 ppm arose from the six C-H protons, associated with the $C_2$ to $C_6$ carbons in the glucuronic acid ring.

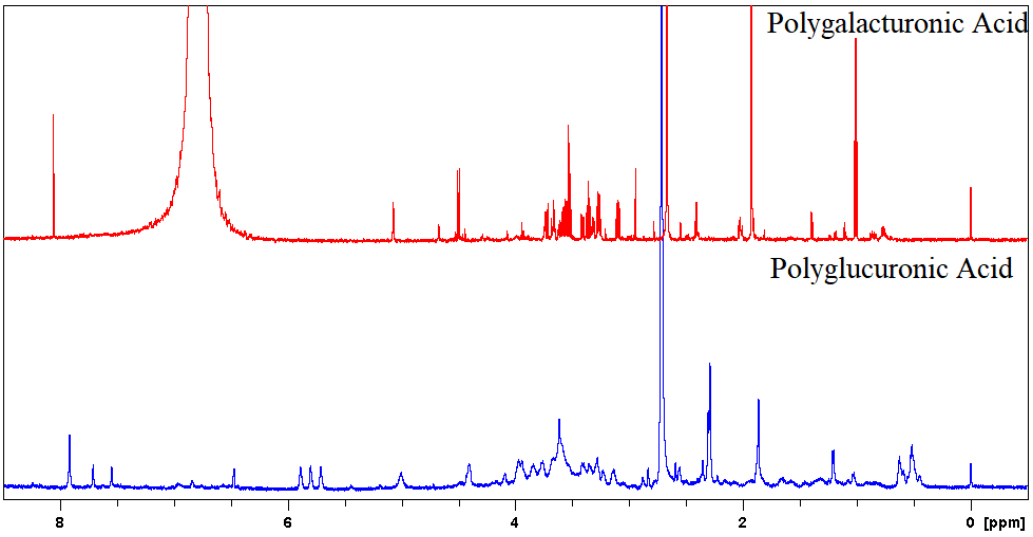


**Fig. 2.** $^1$H-NMR spectra of the hydrolysates of the polygalacturonic and polyglucuronic acids measured in 72% $D_2SO_4/D_2O$

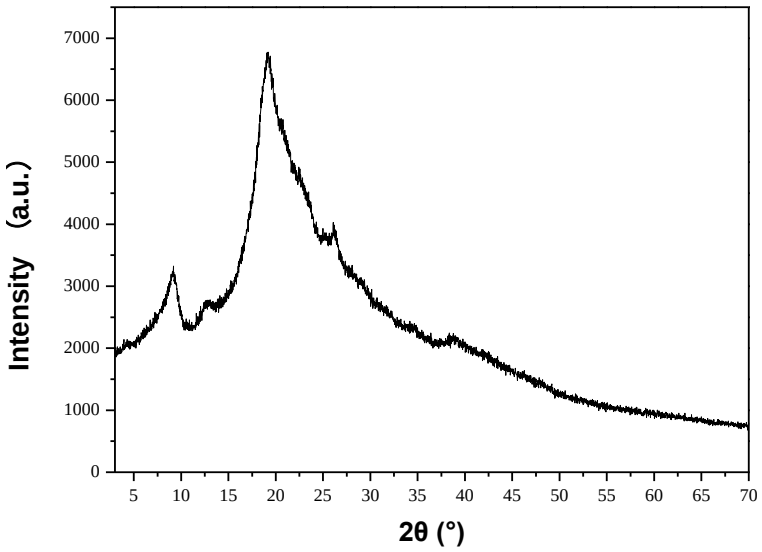


**Fig. 3.** XRD patterns of the polyuronic acid

Due to the high crystallinity of the synthesized polyuronic acid, it is difficult to dissolve them in $D_2O$, the quality of $^1$H-NMR spectra are not so good. The peaks at 5.1 to 5.2 ppm were concluded as β glycosyl linkage of 1,4 polygalacturonic and polyglucuronic acids. Figure 3 shows that the synthesized polyuronic acid had amorphous XRD patterns. It indicated that certain amorphous phase in the polymer sample along with crystalline phase, leading to such broad peaks. The degree of crystallinity was calculated to be 59.3% by peak intensity method.

## CONCLUSIONS

Polyuronic acid is widely used in the fields of improving immunotherapy and moisturizing cosmetics. Up to now, the synthesis process has been complex and costly. The study improved a green method to synthesize polyuronic acid using *A. xylinum*. The structural features of the two polyuronic acid samples were characterized by GPC, FTIR, $^1$H-NMR, and XRD analyses. The Mw values of the polyglucuronic acids reached 150000 to 390000 and the D values were approximately1.01 to 1.35. The FTIR and $^1$H-NMR spectra indicated that glycosidic linkages were successfully formed. Compared with the previous reported synthesis of polyuronic acid by TEMPO oxidation, this method has advantages in both environmental and ecological costs.

## ACKNOWLEDGMENTS

The authors are grateful for the support of the National Key Basic Research Program of China (Project No. 2017YFD0601005). The work was also supported by the Nanjing Forestry University Outstanding Youth Fund (NLJQ2015-5) and the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD), China.

## REFERENCES CITED

Delattre, C., Pierre, G., Gardarin, C., Traikia, M., Elboutachfaiti, R., Isogai, A., and Michaud, P. (2015). "Antioxidant activities of a polyglucuronic acid sodium salt obtained from TEMPO-mediated oxidation of xanthan," *Carbohyd. Polym.* 116, 34-41. DOI: 10.1016/j.carbpol.2014.04.054.

Elboutachfaiti, R., Delattre, C., Petit, E., and Michaud, P. (2011). "Polyglucuronic acids: Structures, functions and degrading enzymes," *Carbohyd. Polym.* 84, 1-13. DOI: 10.1016/j.carbpol.2010.10.063.

Hestrin, S., and Schramm, M. (1954). "Synthesis of cellulose by *Acetobacter xylinum*. 2. Preparation of freeze-dried cells capable of polymerizing glucose to cellulose," *Biochem. J.* 58(2), 345-352.

Heyraud, A., Courtois, J., Dantas, L., Colin-Morel, P., and Courtois, B. (1993). "Structural characterization and rheological properties of an extracellular glucuronan produced by a *Rhizobium meliloti* M5N1 mutant strain," *Carbohyd. Res.* 240, 71-78. DOI: 10.1016/0008-6215(93)84172-3

Isogai, A., and Kato, Y. (1998). "Preparation of polyuronic acid from cellulose by TEMPO-mediated oxidation," *Cellulose* 5(3), 153-164. DOI: 10.1023/A:1009208603673

Jacob, J., Haponiuk, T. J., Thomas, S., and Gopi, S. (2018). "Biopolymer based nanomaterials in drug delivery systems: A review," *Mater. Today Chem.* 9, 43-55. DOI: 10.1016/j.mtchem.2018.05.002

Ray, B. (2006). "Polysaccharides from *Enteromorpha compressa*: Isolation, purification and structural features," *Carbohyd. Polym.* 66(3), 408-416. DOI: 10.1016/j.carbpol.2006.03.027

Ray, B., and Lahaye, M. (1995a). "Cell-wall polysaccharides from the marine green alga *Ulva "rigida"* (ulvales, chlorophyta). Extraction and chemical composition," *Carbohyd. Res.* 274, 251-261. DOI: 10.1016/0008-6215(95)00138-J

Ray, B., and Lahaye, M. (1995b). "Cell-wall polysaccharides from the marine green alga *Ulva "rigida"* (ulvales, chlorophyta). Chemical structure of ulvan," *Carbohyd. Res.* 274, 313-318. DOI: 10.1016/0008-6215(95)00059-3

Tavernier, M. L., Petit, E., Delattre, C., Courtois, B., Courtois, J., Strancar, A., and Michaud, P. (2008). "Production of oligoglucuronans using a monolithic enzymatic microreactor," *Carbohyd. Res.* 343, 2687-2691. DOI: 10.1016/j.carres.2008.06.002

Article submitted: July 28, 2018; Peer review completed: September 17, 2018; Revised version received: September 26, 2018; Accepted: September 28, 2018; Published: October 2, 2018.
DOI: 10.15376/biores.13.4.8646-8652