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Biosynthesis of Low Relative Molecular Mass Bacterial Cellulose in Presence of D-xylose

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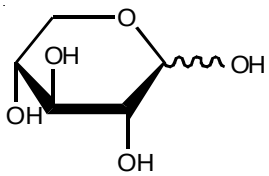
Acetobacter xylinum strain was screened in our own laboratory. It can used several saccharides as carbon source. Coconut water was adapted as incubating system. Bacterial cellulose was biosynthesized by static fermentation culture. Low relative molecular mass bacterial cellulose was obtained when D-xylose was added into the medium, which water-holding capacity, structure features, crystallization properties, thermal decomposition behaviours of the bacterial cellulose almost have no difference compared with pure bacterial cellulose. But the molecular mass of product is lower than that of pure bacterial cellulose. D-xylose maybe used as sealing carbon source.

Keywords: Low relative molecular mass bacterial cellulose, Biosynthesis, D-xylose.

INTRODUCTION

Bacterial cellulose (BC), one kind of exopolysaccharides secreted by some microorganisms, is the principal constituent of the gelatinous membrane formed in the fermentation broth. Acetobacter xylinum is the highest bacterial cellulose production and yield much higher than plant cellulose. Bacterial cellulose possesses some outstanding properties comparing with plant cellulose, such as high purity and crystallinity, high modulus and tensile strength, excellent water holding capacity and biocompatibility. The important is that the shape and some performance of bacterial cellulose can be adjusted during its process of biosynthesis. It's regarded as a good model compound for studying cellulose [1-3]. Therefore it may be the best cellulose in the world at present.

The degree of polymerization (DP_W) of bacterial cellulose was about 1050-16800 [4,5]. Though a few literature reported that bacterial cellulose can be dissolved in some ionizing solvent [6], according to our research work, bacterial cellulose is very difficult to dissolve even if in ionizing solvent. It's hard to get effective information of its structure when using the ionic solution of bacterial cellulose to test NMR due to the interference of solvent peaks. Moreover, we will especially need dissolvable bacterial cellulose whose relative molecular mass is standard and the molecular mass distribution of which should also be narrow even homogeneous. So, to prepare dissolvable bacterial cellulose of different relative molecular mass, it's necessary to seek a way of controlling the relative molecular mass of bacterial cellulose. Furthermore, in the process of the biosynthesis of high molecular mass poly-β-hydroxybutyrate (PHB), adding some monomers, such as glycerol [7] which can prevent the propagation of the molecular chain for blocking. In the present study, imagining a way of controlling the relative molecular mass imitating the control of poly-β-hydroxybutyrate, D-xylose as carbon source was added which can prevent the propagation of the molecular chain as end capping agent when synthetizing bacterial cellulose, these substrates can be converted to be a part of production directly so that the molecular chain of bacterial cellulose can't be propagating. And mainly study the links between adding amount and the molecular mass and structure of the products.



Structure of D-xylose

EXPERIMENTAL

Microbial strain: Acetobacter xylinum selected by our laboratory; Coconut-water: Hainan local coconut-water, 3-5 days' natural fermentation; D-xylose (AR, Aladdin reagent) and other reagents are all analytical reagent.

Agar slant culture-medium (g/L): Ammonium sulfate 3-5, magnesium sulfate 0.2-1, agar 15-20, fresh coconut-water; pH = 3.5-5.0. High temperature sterilization, 20 min.

Seed culture medium (g/L): Sucrose 0-50, ammonium sulfate 3-5, magnesium sulfate 0.2-1, potassium dihydrogen phosphate 0.5-2, naturally fermented coconut-water; pH = 3.0-5.0. High temperature sterilization, 20 min.

Fermentation medium (g/L): Sucrose 0-50, ammonium sulfate 3-5,magnesium sulfate 0.2-1, potassium dihydrogen phosphate 0.5-2, naturally fermented coconut-water; pH = 3.0-5.0. High temperature sterilization, 20 min.

Fourier transform infrared spectrometer (T27): Germany Bruker company; X-ray diffractometer (D8 Advance): Germany Bruker company; Gel Permeation Chromatography (Waters1515 HPLC pump, Waters 2414 RI detector): America Waters company; Scanning electron microscope (S-3000N): Japanese Hitachi company; Integrated thermal gravimetric analyzer (SDT-Q600): America TA company.

Pellicles production and purification: Took strains which have been cultured on agar slant culture-medium with a inoculating loop and give a static culture in the seed culture medium under the condition of 30 °C for 2 days. The culture vessel was filled with 150 mL fermentation media and 5 % inoculation amount, which was static cultured 3 days under the condition of 30 °C.

In the process of the preparation, add D-xylose with different concentration (0-20 g/L) into fermentation medium respectively.

After 3 days, static culture, there was a gel membrane on the surface of the medium. Took out of the gel membrane and rinsed them for 24 h under rushing water, then the cellulose pellicles were dipped into 500 mL ρ(NaOH) = 10 g/L NaOH solution for 2-4 h boiling to eliminate the cells and components of the culture liquid. The pH was then reduced to 7 with weak acid, finally wash them with distilled water repeatedly and get the gel D-xylose-bacterial cellulose membrane. The membrane was dried under an stable temperature of 80 °C until its weight was unchanged and obtained the drying pellicles.

Yield of D-xylose-bacterial cellulose: Proposing that the weight of a drying pellicle is m (g) and the volume of the pristine culture liquid is V(L). The yield(R) of D-xylose-bacterial cellulose (g/L) is

$$R = m/V \quad (1)$$

Characterization of D-xylose-bacterial cellulose

The structure of the D-xylose-bacterial cellulose was characterized with Fourier transform infrared spectrometer. The SEM with an amplification of 1000 was used to observe the microcosmic structure of D-xylose-bacterial cellulose. An X-ray diffraction analysis was performed to test the crystallinity of D-xylose-bacterial cellulose. Condition of the test: Cu target, pipe pressure of 40 V, pipe flow of 40 mA, scanning range of 5-40°. After fitting the spectra with JADE5.0, calculate the crystallinity index (CrI) as follow formula:

$$\text{Crystallinity index} = \frac{A_{crystal}}{A_{Total}} \times 100 \quad (2)$$

where, $A_{crystal}$ is the peak area of crystalline diffraction peak and A_{Total} is total diffraction peak area in the range of 5-40°.

Shattered the drying pellicles into powder and carried out a thermogravimetric analysis (TGA) with thermogravimetric analyzer to test its thermal property. Test condition: N_2 atmosphere, heating rate was 10 °C/min and the temperature was in a range of 30-600 °C.

Derivatization and gel filtration chromatography analysis: D-xylose-bacterial cellulose powder (0.25 g) was suspended in 2.5 mL trifluoroacetic acid (TFA) and 0.8 mL trifluoroacetic acid anhydride (TFAA) and the mixture was stirred until a highly viscous and clear solution was obtained (usually 2-3 h). The solution was diluted with 6 mL chloroform. Then kept at room temperature for 16 h. Precipitation upon addition of 200 mL diethyl ether, washing with diethyl ether and drying for 20 h at 80 Pa and room temperature yielded a white polymer. These samples still contained trifluoroacetic acid and diethyl ether. The removal of the impurities was by thermal treatment under reduced pressure (40 min, 150-80 Pa). then we obtained purified D-xylose-bacterial cellulose trifluoroacetate (CTFA). 0.3 g CTFA was dissolved in 1 mL tetrahydrofuran (THF) which is chromatographically pure. Sampled after filtration. The chromatographic column was polystyrene gel clumn and the standard sample was polystyrene. The column temperature was 25 °C, sampling time 15 min at 1 mL min^{-1}.

Water holding rate (WHR) test: After aspirating the water on the surface of the D-xylose-bacterial cellulose with filter papers, the pellicles were defined as wet pellicles and the weight of which is m_1. Dry them under 80 °C until the weight was unchanged, then got pellicles which were called drying pellicles and the weight of which is m_2. Calculate as the follow formula:

$$\text{Water holding rate} = \frac{m_1 - m_2}{m_2} \quad (3)$$

RESULTS AND DISCUSSION

Characterization of D-xylose-bacterial cellulose

FT-IR analysis. Fig. 1 shows the infrared spectrum of pristine bacterial cellulose and D-xylose-bacterial cellulose whose ρ(D-xylose) is 10 and 20 g/L respectively. In contrast to pristine bacterial cellulose, their infrared spectrum shows all the characteristic peaks which are also showed in pristine bacterial cellulose.

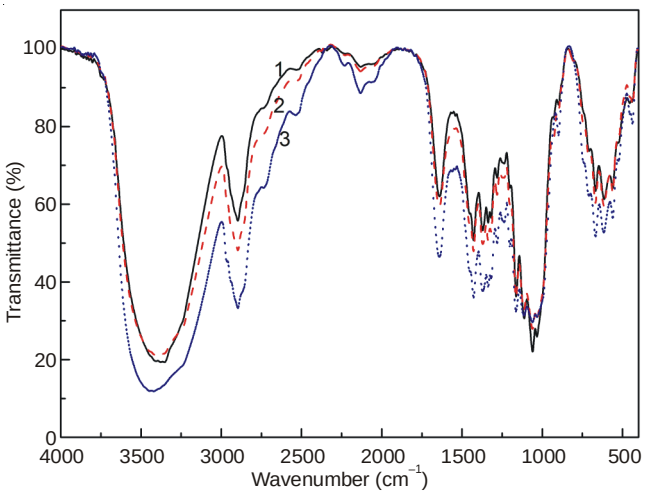


Fig. 1. FTIR spectra of the bacterial cellulose (1: pure bacterial cellulose, 2: 10 g/L D-xylose-bacterial cellulose, 3: 20 g/L D-xylose-bacterial cellulose)

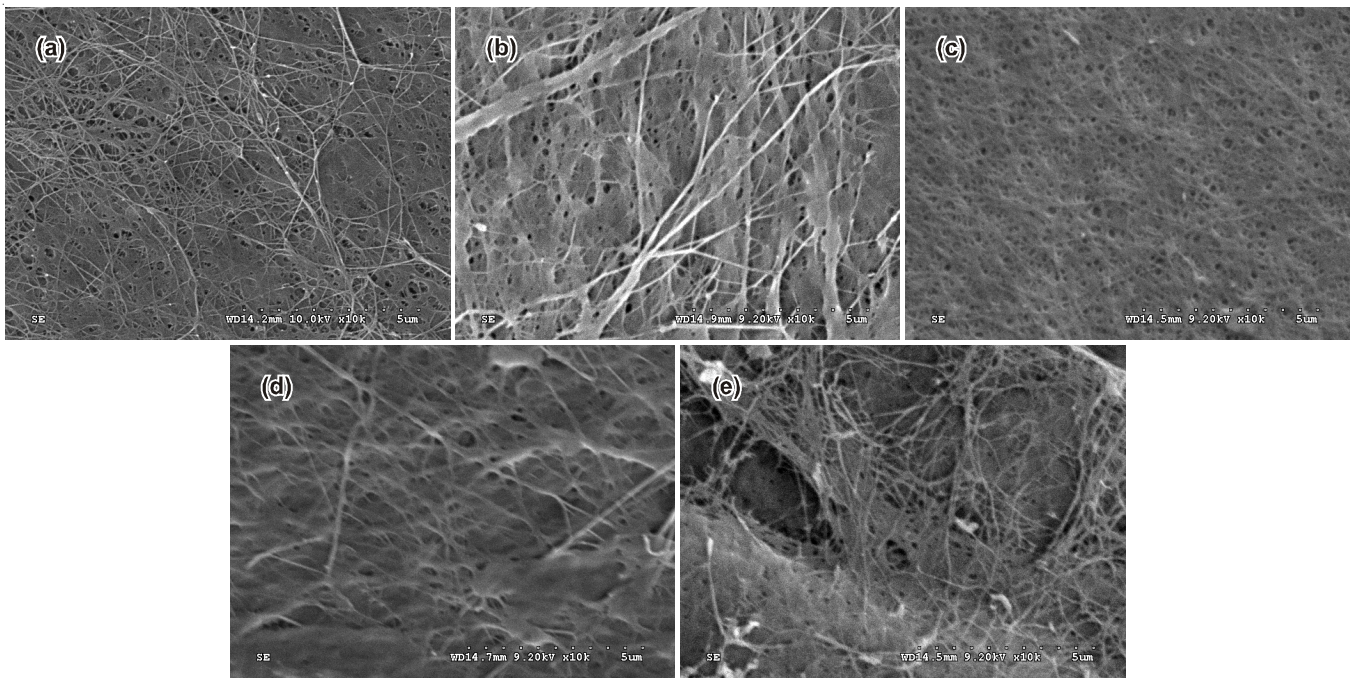


Fig. 2. SEM images of pure bacterial cellulose and xylose-bacterial cellulose powders (a: pure bacterial cellulose, b: 5 g/L D-xylose – bacterial cellulose, c: 10 g/L D-xylose-bacterial cellulose, d: 15 g/L D-xylose – bacterial cellulose, e: 20 g/L D-xylose – bacterial cellulose)

SEM observation: Fig. 2 is the SEM picture of pristine bacterial cellulose and D-xylose-bacterial cellulose with different ρ(D-xylose). It is clearly seen that the three-dimensional network structure of pristine bacterial cellulose. After adding D-xylose, the three-dimensional network structure of the products become more compact and the holes become smaller. Fig. 2b shows the microcosmic structure of the products when the amount of the D-xylose we added is 5 g/L. But three-dimensional network structure showed in Fig. 2c-2e are more compact. It is possible that when there are larger amount of D-xylose-bacterial cellulose being added, the three-dimensional network of bacterial cellulose will be changed and become more ordered, more compact.

X-ray diffraction test: X-ray diffraction spectra (Fig. 3) shows that the intensity is different due to the amount of sample is different, but diffraction peaks are same. There are diffraction peaks at 14.5°, 16.9°, 22.7° and the diffraction peaks reached highest at 22.7°. Firstly, fitting the XRD intensity curve of bacterial cellulose composite with software JADE5.0, then calculate the crystallinity index of the bacterial cellulose according the ratio of the peak area of the crystallinity to total area of the fitting, the crystallinity index was showed in Table-1. With added increasing the ratio of the D-xylose, the crystallinity index of the products was decreasing, then raised up and declined at last. But the variation is not very obvious.

TGA test: From the curve of thermal decomposition, which can easily found that the pattern of thermal decomposition curve varies little. There were two stages. The first stage

TABLE-1
CRYSTALLINITY OF THE PRODUCTS OF DIFFERENT RATE OF SUGAR AND D-XYLOSE

Adding carbon source sucrose:D-xylose (g/L)	20:0	15:5	10:10	5:15	0:20
Crystallinity index (%)	96	87	89	91	87

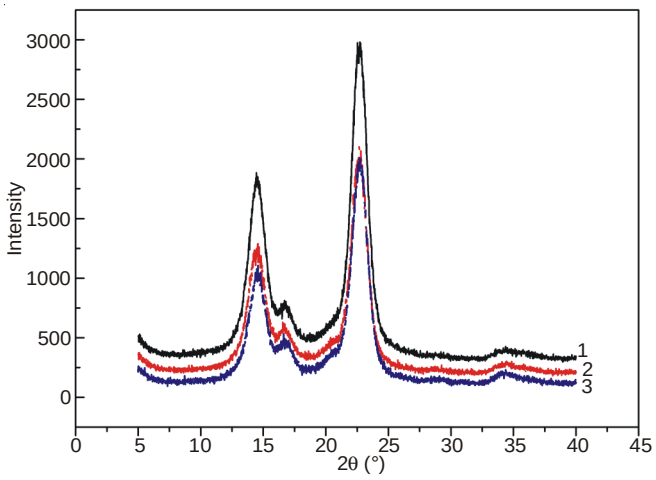


Fig. 3. X-ray diffraction spectra (1: pure bacterial cellulose, 2: 10 g/L D-xylose-bacterial cellulose, 3: 20 g/L D-xylose-bacterial cellulose)

was losing water and the second was the beginning of decomposition at 300 °C or so, then it almost discomposed totally around 600 °C. The thermal stability has been improved after adding D-xylose. The mainly reason may be that after adding D-xylose, the purity of the pellicles is higher, and the impurity was less than before (Fig. 4).

There aren't too obvious distinctions between the maximum weight loss temperature of different products with different ratio of sucrose to D-xylose. It almost didn't vary, which suggest that they were the same substance. Combining the results of FT-IR analysis and TGA, we can preliminarily concluded that the products after adding different ratio of D-xylose and the pure bacterial cellulose was the same substance.

GFC test: D-xylose-bacterial cellulose is one kind of macromolecular compound, usually, we dissolve it in dimethylacetamide/LiCl [8] and test the dissolved solution with Ubbelohde viscometer to test its molecular mass. However, in this way,

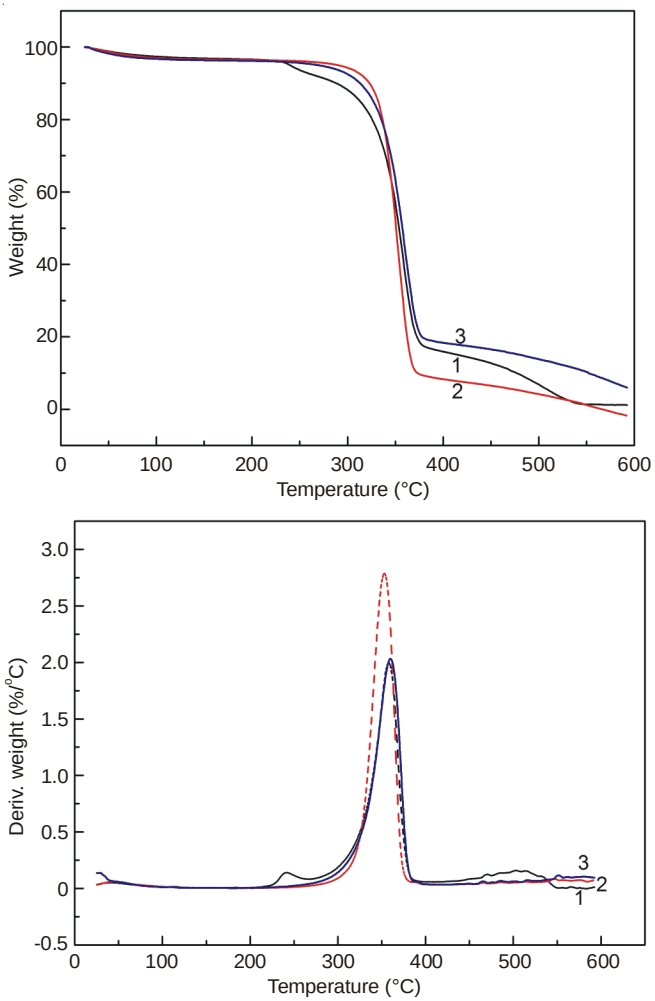


Fig. 4. Thermal decomposition of figure (1: pure bacterial cellulose, 2: 10 g/L D-xylose-bacterial cellulose, 3: 20 g/L D-xylose-bacterial cellulose)

TABLE-2
THERMAL STABILITY OF THE PRODUCTS OF DIFFERENT RATE OF SUGAR AND D-XYLOSE

Adding carbon source sucrose:D-xylose (g/L)	20:0	15:5	10:10	5:15	0:20
Maximum weight loss temperature (°C)	358	363	353	351	359
Maximum loss weight (%)	83	81	90	88	80

we can only detect the molecular mass and polymerization degree roughly. Gel filtration chromatography (GFC) is a common approach to test the molecular mass of macromolecular compound, but it requires the sample can be dissolved in water or organic solvent. However, D-xylose-bacterial cellulose doesn't dissolve in water and common organic solvents. Therefore, we carried out a chemical derivatization on D-xylose-bacterial cellulose and test its molecular mass through testing the molecular mass of the derivative products.

Carry out a chemical derivatization on D-xylose-bacterial cellulose and pristine bacterial cellulose respectively, dissolving in THF, then the results of the GPC test was showed in Table-3. Comparing with the derivative products of pure bacterial cellulose, after adding D-xylose, the relative molecular mass of the derivative products was lower and the index of relative molecular mass distribution didn't vary too much; adding different ratio of D-xylose, the indexes of relative molecular mass of cellulose-trifluoro acetate were almost equal. Thus it can be seen, adding D-xylose can decrease the relative molecular mass of the bacterial cellulose, but there were not too many links between the ratio of D-xylose and the index of relative molecular mass distribution.

Water holding rate test: Three groups of strains were cultured in parallel, calculating the average water holding rate following formula (3). The average water holding rate remained stable at 136 g water/g drying membrane. The water holding rate showed a tendency of decreasing, but it's not obvious (Table-4). This indicate that the products of different ratio of sucrose to D-xylose was the same substance as pure bacterial cellulose in a further step.

TABLE-4
AVERAGE WATER-HOLDING RATE AND YIELD OF THE PRODUCTS WITH DIFFERENT RATE OF SUGAR AND D-XYLOSE

Adding carbon source sucrose:D-xylose (g/L)	Water holding rate (g water/g drying pellicle)	Yield (g/L)
20:0	141	5.4
15:5	136	2.6
10:10	135	2.7
5:15	135	2.8
0:20	131	2.6

Table-4 showed that the yield of the products was highest when the ratio of sucrose to D-xylose is 20:0; when the adding amount of D-xylose was 5, the yield of the products decreased; when the adding amount increased from 5 to 15, the yield of the products showed tendency of increasing; when adding amount of D-xylose was 20, strains cannot converted most D-xylose into intermediate metabolite, therefore the yield decreased again.

TABLE-3
MOLECULAR WEIGHT AND MOLECULAR WEIGHT DISTRIBUTION OF CTFA CELLULOSE OF THE PRODUCTS WITH DIFFERENT RATE OF SUGAR AND XYLOSE IN THE CULTURE MEDIUM

Adding carbon source sucrose: D-xylose (g/L)	Relative molecular weight of cellulose-trifluoro acetate				Index of molecular weight distribution
	Number average molecular weight (M_n)	Weight average molecular weight (M_w)	Peak molecular weight (M_p)	Z average molecular weight (M_z)	
20:0	335673	347662	344861	359674	1.04
15:5	179290	229122	253538	276337	1.28
10:10	191881	236765	233761	279297	1.23
5:15	182324	228092	272965	271786	1.25
0:20	206268	249748	230335	289036	1.21

Conclusions

• Choosing coconut-water as the main culture system, adding different ratio of D-xylose into culture medium in the process of the biosynthesis of bacterial cellulose, the infrared spectrum, crystallization property, the maximum thermal decomposition temperature, the maximum percentage of weight loss and water holding rate of the products were almost same as those of pristine bacterial cellulose.

• After adding D-xylose, the three-dimensional structure of products became more compact, the holes were smaller, the three-dimensional network structure of products became more ordered and more compact.

• After adding D-xylose, both the yield and the relative molecular mass of the products decreased.

ACKNOWLEDGEMENTS

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