Preparation of Xylooligosaccharides from Cotton Seed Huskby *Sphingomonas paucimobilis*

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Abstract: As a kind of agricultural waste, cotton seed husk is a potential source of functional xylooligosaccharides. In this study, a new strain NS5, which could produce endo-xylanase, was screened by France Biomerieux microorganism automatic identification system and preliminarily identified as *Sphingomonas paucimobilis*. The optimum enzymolysis conditions for xylooligosaccharides production were obtained as follows: temperaure 30 ℃, time 8 h, xylanase concentration 15 % and xylan concentration 40 g/L. Under these conditions, the yield of xylooligosaccharides was 53.20%. By analysis with HPLC, xylooligosaccharides was accounted for more than 82% of the total sugar, 48.56 % of which were identified as xylobiose and xylotriose. These results provided information for xylooligosaccharides preparation from cotton seed husk for future use in food industry.

Key words: cotton husk; *Sphingomonas paucimobilis*; screening; enzymatic hydrolysis; xylooligosaccharide; xylose

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利用少动鞘氨醇单胞菌酶解制备棉籽壳低聚木糖

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摘要：本文研究了利用自筛菌株酶法制备棉籽壳低聚木糖的基本工艺。低聚木糖是主要的功能性食品添加剂，棉籽壳是生产低聚木糖的良好来源。因此，如何有效的从棉籽壳中提取低聚木糖成为亟待解决的问题。本研究中通过筛选鉴定（法国梅里埃生物自动识别系统）得到一株新的产内切型木聚糖酶的菌株-少动鞘氨醇单孢菌。通过酶解木聚糖工艺的优化，结果表明：当酶解温度为 30 ℃，酶解 8 h，木聚糖酶的浓度 15%，底木聚糖浓度为 40 g/L 时，低聚木糖的得率可达到 53.20%，经 HPLC 分析，酶解野种木二糖和木三糖占低聚木糖总量的 48.56%，低聚木糖占总糖的 82%以上，以上研究可为工业生产低聚木糖工艺的优化提供依据。

关键词：棉籽壳；菌株；筛选；酶法水解；低聚糖；木糖

Cotton seed husk is a widely distributed and abundant agricultural waste in China with an annual generation rate of 200 million tons. It is a rich source of cellulose (37~48%), hemicellulose (22~28%), lignin and a wide varieties of procyanidins, has received increasing attentions for their recognized health benefits[1]. Xylans, as the most abundant hemicelluloses of land plants, are the α-1,4-linked polymers of xylopyranosyl units with a degree of polymerization ranging from 70 to 200[2]. Thus, it could be an appropriate primary material for the production of xylooligosaccharides.

Xylooligosaccharides play a crucial role in keeping the balance of bacteria in the intestine by increasing the number of *bifidobacteria* and *lactobacilli* and suppressing the growth of *Clostridium*[3]. They also cause prebiotic effects after being ingested through the modulation of colonic microflora. Meanwhile, they are stable in acidic media, resistant to heat, and able to offer lower available energy and achieve significant biological effects at low daily intake. So far, xylooligosaccharidesare usually prepared from xylan-containing lignocellulosic materials by chemical methods, direct enzymatic hydrolysis of a susceptible substrate[7] or a combination of chemical and enzymatic treatments and followed by the refining step to remove both monosaccharides and non-saccharide compounds[4,5].

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Nevertheless, there were less reports on optimization of xylooligosaccharides production by enzymolysis and from cotton seed husk.

In the previous study, we had used the cotton seed husk to prepare the functional xylooligosaccharides. The objective of this study was to isolate xylanase producing strains and optimize the conditions for xylooligosaccharides preparation to make full use of cotton seed husk.

1 MATERIALS AND METHODS

1.1 Materials and chemicals

Cotton seed husks were obtained from Green Ecological Laboratory of Shihezi University, and crude cotton husk xylan (62.8%) prepared in laboratory. All the chemicals used in the study were of analytical grade or superior grade.

1.2 Pretreatment of cotton seed husk

The cotton seed husk was cleaned and dried up, then homogenized to 60 meshes. The powder was soaked in 95% alcohol overnight. The filtrate was removed by vacuum filtration and flushed with the deionized water, then adjusted to pH 7.0. Finally, about 40 g of the above-mentioned cotton seed husk was digested under the reported conditions (digestion temperature 115 ℃, digestion time 45 min and addition of water 0.6 L) and centrifuged at 4500 r/min for 10 min (4 ℃).

1.3 Bacterial strain screening for xylanase production

Strains of C, M, L, NS were isolated from soil samples, collected from Shihezi Southern Mountain and were maintained in medium I as follows: 2.0% cotton seed husk powder, 0.3% $(NH_4)_2SO_4$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.05% $FeSO_4$. Samples from soil in sterilized water were poured and spread onto agar plate A (medium: 1.5% cotton seed husk xylan, 0.2% yeast extract, 1.8% agar, 0.03% $(NH_4)_2SO_4$, 0.1% K_2HPO_4, 0.05% $MgSO_4$). These plates were incubated at 37 ℃ for 24 h. The colonies on the plates were transferred onto agar plate B, which were again incubated at 37 ℃ for 24 h. Colonies producing clear zone in the plate were selected. To further identify the bacteria strain, the second screening for bacteria strains was conducted by shake flask cultivation. The medium components for shake flask cultivation were 1 % crude xylan, 0.2% yeast extract, 0.2% $(NH_4)_2SO_4$, 0.1% K_2HPO_4, 0.05% $MgSO_4$ and 0.001% $FeSO_4$. The cultivation conditions for bacteria strain were: rotating speed 220 r/min, temperature 30 ℃, time 48 h. The enzyme activity as evaluation index was determined by the previous reported DNS method [2]. The optimal strains were selected by assay of enzyme activity and Tthin layer chromatography (TLC) was used for identification. The TLC test was conducted according to the previous reports [6].

1.4 Identification of bacterial strain

The isolated bacteria was cultured at 30 ℃ for 48 h, made suspension in a certain concentration with physiological saline solution subsequently, and identified by France Biomerieux microorganism automatic identification system VITAK-32.

1.5 Production of xylanase from bacterial strain

The influences of yeast extract (0.1~0.5%), xylan (1~3%) and Tween 80 (0.04~0.08%) concentrations on xylanase activity were investigated. The variable cultural conditions (pH, temperature, quantity of inoculation, rotating speed) which also affected the xylanase activity were determined by single factor test.

1.6 Preparation of crude xylooligosaccharide

The xylan was digested by the xylanase produced from the optimal bacterial strain. The crude xylan (5 g for each) was mixed with 100, 200, 300, 400 and 500 mL water to a final concentration of 10~50 g/L. The pH (8.0) of suspension kept constant by adjusting it with 0.5 M NaOH or 0.5 M HCl. Then added the prepared xylanase to the suspension with a concentration 0~20% (*m*/*V*) and digested it under the following conditions: temperature 25~40 ℃, time 2~10 h. After the enzymolysis, the sample was boiled for 5~10 min to deactivate the enzyme. The mixture was centrifuged at 8000 r/min for 10 min and then the supernatant was collected. The concentration of reducing sugar and total sugar and average degree of polymerization were determined by the reported method respectively [7].

1.7 HPLC analysis of xylooligosaccharides

Xylooligosaccharides was detected by HPLC equipped with a refractive index detector (Waters 600). Before injection, samples were filtered through 0.20 μm filter. Aliquots of filtered sample (20 μL) were injected to the HPLC system. Xylooligosaccharides were eluted

using distilled–deionized water as the mobile phase from an ion-mediated stationary phase in the silver form (Bio-Rad Aminex HPX 42A). The Aminex HPX 42A column (300 mm × 7.8 mm), which was preceded by its complimentary de-ashing cartridge (Bio-Rad), was used at 30 ℃ and a flow rate of 0.6 mL/min. A complete analysis of xylooligosaccharides was carried out in 45 min. Concentration of an oligosaccharide was quantified using average peak areas compared with mixture of standard oligosaccharides (Sigma, X2-X6).

2 RESULTS AND DISCUSSION

2.1 Bacteria screening and xylanase preparation

In order to get the dominant strain for xylanase production, the strains (C, NS, M, L) were screened primarily according to the diameters of transparent zone (Fig.1). There were eight samples in each group. According to the transparency and diameter of transparent zone, ten bacteria strains were selected for further screening (NS4, NS5, C7-11, M5, M11, M11-2, M23, M25, L8, L23-1).

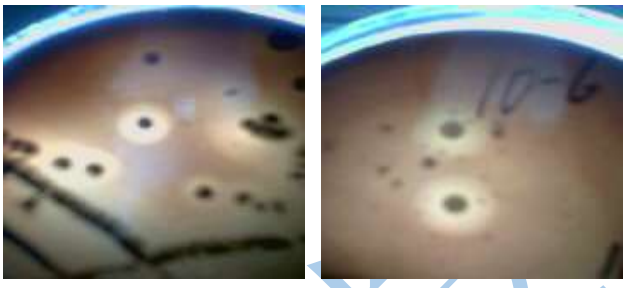

Fig.1 Clarity Circle

To further screen the bacteria strains, the flask cultivation was applied. Through enzyme activity assay, bacteria strains (NS4、NS5、M11-2、M25) were obtained with high production of enzyme and high stability (Table 1).

Table 1 The result of second screening

Strains	NS4	NS5	C7-11	M11	M11-2	M23	M25	M5	L8	L23-1
Enzyme activity/(U/mL)	11.2	14.5	6.8	4.6	17.5	5.8	12.6	7.7	8.6	6.3

The enzyme activity of strain M11-2 was 17.5 U/mL, which was higher than strain NS5. Afterwards, TLC was employed to elucidate whether the above four bacteria strains could produce endo-nucleases. Through TLC detection, only the NS5 strain produced endo-xylanase (data not shown) that hydrolyze xylan with high specificity (Fig.2). The components of hydrolysed xylan were mainly xylobiose and xylotriose, and few xylan and arabinose were observed. Therefore, the NS5 strain was responsible for xylanase production, which was identified as *Sphingomonas paucimobilis* subsequently by France Biomerieux microorganism automatic identification system VITAK-32.

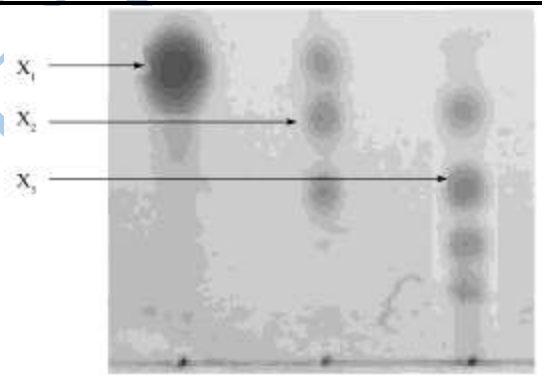

Fig.2 The production of xylan through enzymatic hydrolyzes by TLC

Note: X_1: The TLC test results of the xylose standard solution (1%); X_2: The TLC test results of NS5 enzymatic hydrolysate; X_3: The TLC test results of the oligosaccharides standard solution (1%).

High xylanase activities and limited xylan degradation at the early stages of biodegradation were also recently reported when *C. subvermispora* and several other wood-decay fungi were grown on *Eucalyptus* grandis[8, 9]. Many microorganisms were known to produce more than one xylan-depolymerizing enzyme. Heidorne et al[10] found that *C. subvermispora* produced at least three xylanases and two mannanases. Although many mesophilic fungal xylanase had been studied, the strain identified as *Sphingomonas paucimobilis* for xylanase production had not been report before.

2.2 Optimization of xylanase from bacterial strain

The cultivation conditions play significant roles in enzyme production, such as carbon source, nitrogen source and surface active agent and so forth. In the study, about nine combinations of three variable factors were conducted. The optimal medium components were 0.3% yeast extraction, 2% crude xylan and 0.08% Tween 80. The yeast extract was the most important factor for enzyme activity. Meanwhile, the cultivation conditions (pH, temperature, quantity of inoculation, rotating speed) were also studied on the basis of the above optimal

medium (data not shown). Considering about all factors, the cultivation conditions were determined as follows: initial pH 8.0, cultivation temperature 31 ℃, rotating speed190 r/min and quantity of inoculation 10%.

2.3 Optimization of crude xylooligosaccharide preparation

After xylan was isolated from cotton seed husk, the enzymatic hydrolysis conditions for production of xylooligosaccharides were investigated (Fig.3). When xylanase was reacted with xylan, the endonucleases usually acted on β-1, 4 - glycosidic bonds from the inside of main chain of xylan to cut the long-chain xylan into xylooligosaccharides with different length randomly, and then the β-1, 4 - xylosidases acted on the end of xylooligosaccharides to generate xylose[11, 12]. After the enzymatic reaction of 8h, the concentration of total sugar reached to 3.1 mg/mL, but then the concentrations of total sugar and xylose decreased because of the Maillard reaction between the reducing sugar in the sugar solution and the residue protein in the substrate xylan. Therefore, the reaction time of the endo-xylanase to make the concentration of total sugar reaching the highest point was 8 h, which was well consistent with the xylooligosaccharide from bagasse[11].

The concentrations of the total sugar and the xylose increased with the increasing quantity of xylanase (Fig.3). As the concentration of xylanase increased from 5% to 10%, the increase of the concentration of total sugar was larger than that of xylose. Meanwhile, with the increase of the quantity of xylanase, the rate of enzymatic hydrolysis reaction was accelerated to cut the long-chain xylan into short-chain xylooligosaccharides rapidly. Nonetheless, the accumulation of products slowed the speed of enzymatic hydrolysis reaction, and promoted the generation of xylose. The similar results had been reported by Saleh et al[12,13]. Therefore, the ratio of xylooligosaccharides to total sugar was 68.3 % with the xylanase concentration of 15%.

The xylan concentration had crucial influence on the indexes. Fig.3 indicated that the concentration of total sugar and xylose increased obviously with the increase of the substrate xylan. The increase of the concentration of xylan accelerated the rate of enzymatic hydrolysis reaction to cut the long-chain xylan into short-chain xylo-oligosaccharides. Then the increasing xylooligosaccharides created favorable conditions for the generation of xylose, which resulted in the accumulation of xylose. Therefore, the concentration of xylan chosen for the preparation of xylooligosaccharide was 40 g/L, which was approximately 2 times of the reported bagasse and cotton stalks[2,11].

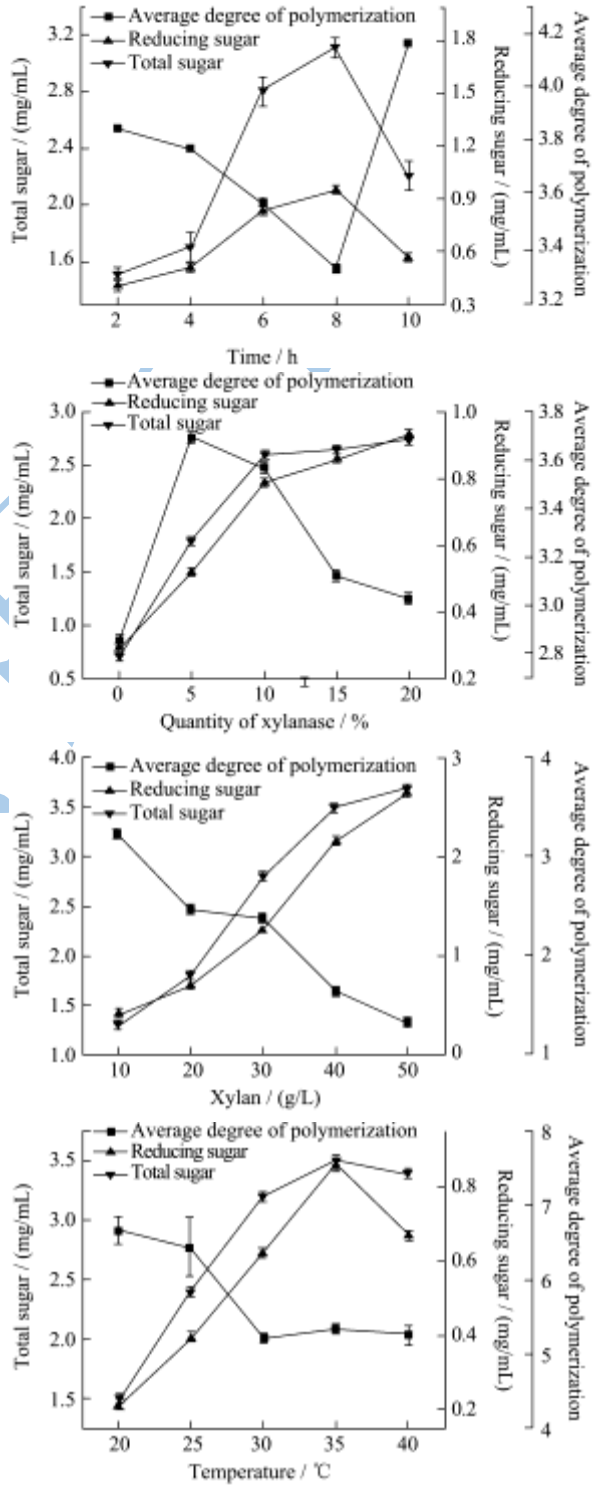

Fig.3 The effects of enzymolysis time, quantity of xylanase, xylan concentration and enzymolysis temperature on xylooligosaccharide preparation

As the temperature of the hydrolysis reaction was increased, the hydrolysis rate and yield also increased, as expected (Fig.3). However, the differences among 30, 35, and 40 ℃ were not very significant, most probably due to thermal instability of the enzyme and the side reaction[2]. When the temperature exceeded 30 ℃, the Maillard reaction was accelerated to consume the reducing monosaccharides, which would reduce the concentration of total sugar and xylose. Overall, the proper temperature for enzymatic hydrolysis was 30 ℃, which was lower than the reported result [2,14].

Based on these observations, the four variables all had obvious effects on the indexes. From the above test, the proper conditions for xylooligosaccharide preparation were obtained, which provided the important support for the following test.

2.4 Xylo-oligosaccharide identification

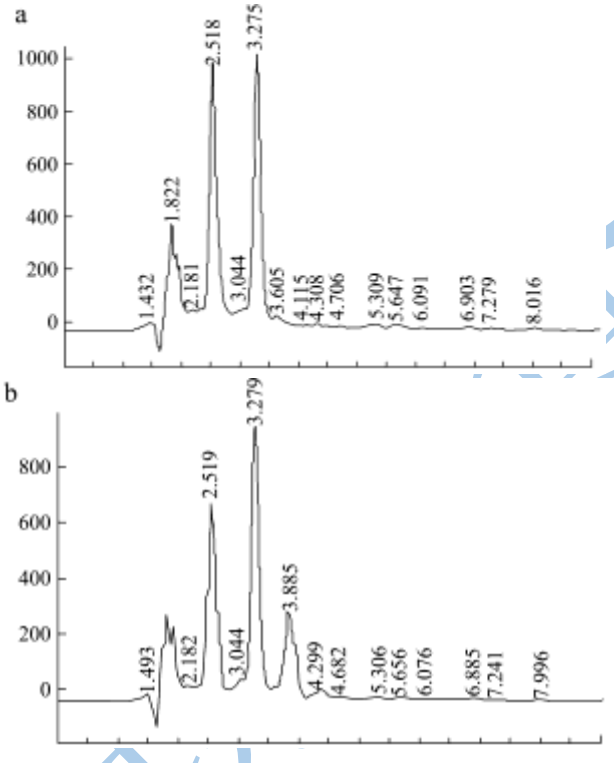

Fig.4 HPLC of xylooligosaccharides

注：a. HPLC of standard mixture of xylooligosaccharides, b. HPLC of xylooligosaccharides。

The purified xylooligosaccharide was identified by HPLC to shed light on the components of it. Pure xylooligosaccharide was as standard sample and from the Figure 4a saw that the retention time for xylobiose and xylotriose was 2.518 min and 3.275 min, respectively. Other peaks showed the retention times of the xylotetrose, the xylopentose, the xylohexose, the xyloheptose, and above. And Figure 4b showed that the retention time for two predominant peaks was 2.519 min and 3.279 min, which was almost the same with the characteristic peaks of xylobiose and xylotriose. Comparing with Fig.4a, we could get that xylobiose and xylotriose accounted for 48.56% of xylooligosaccharides and they accounted for more than 82 % of the total sugar.

3 CONCLUSIONS

This research demonstrated that cotton husk, which had less economical value, could be converted by enzymatic hydrolysis to a more valuable xylooligosaccharide product without production of significant quantities of xylose. Strains for xylanase production had been screened and identified as *Sphingomonas paucimobilis*, which had not been reported before. The optimal conditions for xylooligosaccharide preparation and purification were obtained. The xylobiose and xylotriose accounted for 48.56% of the purified xylooligosaccharides which accounted for more than 82% of the total sugar.

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