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Fermentation Performance and Structure Characteristics of Xanthan Produced by *Xanthomonas campestris* with a Glucose/Xylose Mixture

Zhiguo Zhang · Hongzhang Chen

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Abstract The ability of *Xanthomonas campestris* to convert glucose and xylose to xanthan and the structure of xanthan derived from the glucose/xylose mixture media are important when the lignocelluloses hydrolysate was used in xanthan production. In this paper, the features related to xanthan fermentation in the glucose/xylose mixture media and the structures of xanthan derived from the mixture media were studied. Glucose was the preferred carbon source to produce xanthan while xylose was also utilized with a very low consumption rate. When the fraction of glucose decreased from 100% to 25%, the glucose consumption rate and xanthan production rate reduced from 0.44 g L^{-1} h^{-1} to 0.25 g L^{-1} h^{-1} and 0.21 g L^{-1} h^{-1} to 0.04 g L^{-1} h^{-1} respectively while xylose was consumed at a very stable rate (0.053–0.060 g L^{-1} h^{-1}). On the other hand, when the xylose fraction increased from 0% to 50%, pyruvate and acetate content of xanthan increased from 2.43% to 3.78% and 2.55% to 7.05%. The existence of xylose also led to higher average molecular weight. Therefore, it could be concluded that xylose was not efficiently utilized by *X. campestris* to produce xanthan. The concentration of glucose rather than the total sugar was the main factor to determine the xanthan production. But xylose was helpful to improve the quality of xanthan.

Keywords Xanthan gum · Glucose · Xylose · *Xanthomonas campestris* · Molecular structure · Lignocellulose

Introduction

Xanthan gum, a microbial biopolymer secreted by *Xanthomonas* bacteria, has wide range of applications, such as suspending, stabilizing, thickening, and emulsifying agent in food industry and oil drilling agent in petroleum production [1, 2]. Generally, the carbon sources used for xanthan fermentation are glucose [3], sucrose [4, 5], and starch [1]. Because of the rise in grain price, recently, scientists hope that fermentable sugars derived from

Z. G. Zhang · H. Z. Chen (✉)
National Key Laboratory of Biochemical Engineering, Institute of Process Engineering,
Chinese Academy of Sciences, Beijing 100190, People's Republic of China
e-mail: hzchen@home.ipe.ac.cn

lignocelluloses, which is the major source of renewable organic matter and mainly comprises cellulose, hemicellulose, and lignin, can be hydrolyzed [6, 7] and used to produce xanthan and other chemical products [8–11]. However, different from the common carbon sources, the hydrolysate of lignocelluloses mainly contains glucose and xylose. When the hydrolysate is used to produce ethanol and other chemical products, some metabolic engineering approaches have to be done to make the glucose and xylose be utilized efficiently.

The commonly used bacterium in xanthan fermentation is *Xanthomonas campestris* [1]. Just the same as ethanol production, the ability of *X. campestris* to convert the two sugars in hydrolysate to xanthan and the structure of xanthan derived from glucose/xylose mixture is important for an economically feasible process. Some authors have reported that both glucose and xylose could be utilized by *X. campestris*, which metabolize glucose through Entner-Doudoroff pathway and utilized xylose through pentose phosphate pathway after it was converted to xylulose by xylose isomerase [3, 12]. But some researches reported that xylose was an inhibitor of xanthan production [13].

The molecular structure characteristics of xanthan such as acetate content, pyruvate content, and average molecular weight are also important because they determined the rheological properties and applications of xanthan solution. For example, high level of pyruvate content and high molecular weight leads to high viscosity of xanthan solution while native xanthan solution shows lower viscosity than deacetylated xanthan [2, 14]. On the other hand, non-pyruvylated xanthan was more suitable for oil recovery process [15] and when xanthan was mixed with guaran gum, native xanthan–guaran mixtures exhibited a liquid-like behavior, whereas non-acetylated xanthan–guaran mixtures exhibited a gel-like behavior [16]. Now some influencing factors on xanthan molecular structure have been investigated [17–21]: some authors suggested that limiting nitrogen source lead to more pyruvic acid substitution and less acetic substitution [19]; Casas et al. found that low temperature result in higher average molecular weight [18]. However, the influence of xylose on xanthan molecular structure has not been reported.

The aim of this study was to investigate the fermentation process with glucose/xylose mixture and the characteristics of molecular structure (acetate content, pyruvate content, and average molecular weight) of the xanthan produced from the mixture.

Materials and Methods

Microorganism and Media

X. campestris CGMCC 1.1781, obtained from China General Microbiological Culture Collection Center, was used. The bacteria were grown on potato dextrose agar slopes at 30 °C and stored at 4 °C.

A basal medium (yeast extract powder 3 g L^{-1}, NH_4NO_3 1 g L^{-1}, $MgSO_4 \cdot 7H_2O$ 0.25 g L^{-1}, Na_2HPO_4 0.1 g L^{-1}, $CaCO_3$ 3 g L^{-1}) was used as medium in which desired amount of glucose and xylose were added.

Culture Condition

All fermentation experiments were carried out at 30 °C and 220 rpm on a rotatory shaker with 500-mL Erlenmeyer flasks in which 100 mL media and 10 mL inoculum was added, respectively. The bacteria suspension of *X. campestris* with a concentration of about 4.8×10^8 cells per milliliter was used as inoculum. To prepare it, bacterial lawn cultured in the slopes

was washed with sterile water (10 mL per slope) and grinded by glass beads in a sterile Erlenmeyer flasks.

The cell growth experiments were carried out 12 g L^{-1} glucose/xylose mixture using some tested proportions such as 0 g L^{-1} glucose/12 g L^{-1} xylose, 3 g L^{-1} glucose/9 g L^{-1} xylose, 6 g L^{-1} glucose/6 g L^{-1} xylose, 9 g L^{-1} glucose/3 g L^{-1} xylose, and 12 g L^{-1} glucose/0 g L^{-1} xylose.

The xanthan fermentation was developed as follows: bacteria were cultured in the media containing 12 g L^{-1} glucose for 36 h. Then, desired amount of glucose and xylose, which had been packed in paper bags and autoclaved at 115 °C for 30 min and dried at 60 °C for 4 h, was added in the broth. Finally, the broth was cultured again.

All experiments were performed in triplicate and their average values were reported.

Analytical Methods

Biomass concentration was obtained by comparing OD_{560} to a standard correlation between optical density and cell density [12]. All of the samples of broth, which were generally pH 6.5, were adjusted to pH 3 with HCl solution (5 mol L^{-1}) to remove the residual $CaCO_3$ in media. And then, 2 mL of the broth was diluted by a factor of 10 with deionized water. The diluted solutions were centrifuged at 14,000×*g* for 30 min at 5 °C. Then, the precipitates were re-suspended in deionized water and measured with a spectrophotometer.

After the biomass was determined, the remaining broth was precipitated with 3 vol of 95% ethanol. The precipitation was filtrated and dried at 60 °C for 24 h and then weighed to calculated raw polysaccharide concentration. The xanthan concentration was estimated as the difference-value between the concentration of raw polysaccharide and biomass. Glucose and xylose concentrations were estimated by assaying the two sugars in the supernate by high performance liquid chromatography (HPLC, AGILENT, USA) at 35 °C with a refractive index detector and an Aminex HPX-87H column (Bio-Rad). A H_2SO_4 solution (5 mmol L^{-1}) was used as the mobile phase and the flow rate was 0.6 mL min^{-1}.

Xanthan Separation and Structure Analysis

Culture samples were adjusted to pH 3 with HCl solution (5 mol L^{-1}) to remove the residual $CaCO_3$ in broth and diluted by five times with deionized water. The diluted solutions were centrifuged at 14,000×*g* for 30 min. Then the xanthan was obtained by precipitating the supernatant with three volumes of 95% (*v*/*v*) ethanol and filtrated. The precipitate was freeze-dried and milled into powder for the further analysis.

The values of acetate and pyruvate contents of xanthan molecular were measured by HPLC after these contents were separated from the side chains of xanthan. Before analysis, xanthan powder was dissolved at a concentration of about 0.5 g L^{-1} in deionized water. The exact concentrations of xanthan were determined according to phenol/sulphuric acid method [22]. To prepare the samples for pyruvate analysis, 1 mL of the solution was mixed with 1 mL 0.1 mol L^{-1} phosphoric acid and then the mixture was sealed and placed in a metal heating block. After it was maintained at 90 °C for 90 min, the mixture was made up to 3 mL with water. To prepare the samples for acetate analysis, 1 mL of the xanthan solution was mixed with potassium hydroxide solution (0.2 mol L^{-1}, 1 mL) and then sealed. After flushed with nitrogen gas, the mixtures were maintained at 45 °C for 6 h, made acidic with phosphoric acid, and diluted to 3 mL with water [23]. The condition of HPLC method to determining acetate and pyruvate acid was the same as that of glucose and xylose analysis but an UV detector setting at 210 nm was used.

The differentia of the average molecular weight of polymers was estimated by comparing the intrinsic viscosity ([η]). Mark–Houwink equation describes the dependence of the intrinsic viscosity of a polymer on its molecular weight. It has the form [24]:

$$[\eta] = K \cdot Mr^{\alpha}$$

Where, [η] is the intrinsic viscosity. K and α are constants, which depend on the nature of polymer and solvent as well as temperature. Mr is usually one of the relative molecular mass averages. Hence, there is a positive correlation between [η] and Mr. The value of [η] was measured with an Ubbelohde-type Viscometer (SUNLEX, CHN) using 1 g L^{-1} xanthan gum in 0.1 g L^{-1} NaCl water solution at 25 °C [25].

Rheological properties were determined using a DV-III UL TRA rheometer (Brookfield, USA). The measurement were carried out at 25 °C by using 2 g L^{-1} xanthan solution which was dissolve in 0.1 mol L^{-1} NaCl water solution.

Results and Discussion

Cell Growth on Glucose/Xylose Mixture

To investigate the growth of *X. campestris* in glucose/xylose mixture, the bacteria was cultured in the media containing different ratios of glucose and xylose. As reported by previous research, generally, the ratio of cellulose to hemicellulose of lignocelluloses was between about 2:1 and 3:2. For example, in switchgrass, corn stover, aspen, and rice straw, reported by Gong et al. [26] and Jin et al. [27], the ratios were 45:31, 41:21, 46:26, and 34.8:24.9, respectively. In hydrolysate, the ratio of glucose to xylose is higher because the hemicellulose is easy to degrade by certain pretreatment. For example, William et al. [28] reported that in the hydrolysate of corn stover blade which was treated with cellulase and ferulic acid esterase, the ratio of glucose to xylose was 141.3:30.8; in the hydrolysate of corncob which was pretreated by H_2SO_4 and hydrolyzed by cellulase and cellobiase, reported by Chen et al. [29], the concentrations of glucose and xylose were 51.2 g L^{-1} and 6.8 g L^{-1}, respectively. Hence, in our study, three ratios of mixture was chosen including 3:1 (9 g L^{-1} glucose and 3 g L^{-1} xylose), 1:1 (6 g L^{-1} glucose and 6 g L^{-1} xylose), and 1:3 (3 g L^{-1} glucose and 9 g L^{-1} xylose).

Figure 1 described the growth profiles. The highest and the lowest growth rate were found in the media containing 100% glucose and 100% xylose, respectively. When the fraction of glucose was more than 50%, the growth rates at exponential growth phase were approximately similar. However, when the glucose fraction was below 50%, the growth rates reduced significantly with the increase of xylose proportion. Figure 1 also showed that in glucose media and mixture media, cell growth ceased after 36 h.

The results indicated that xylose could be utilized as carbon source by *X. campestris* at bacterial growth phase but glucose was the preferred carbon source. Though xylose inhibited the cell growth, the influence was not significant when its fraction in glucose/xylose mixture was below 50%.

Xanthan Production from the Glucose/Xylose Mixture

Xanthan production was partially correlated with cell growth and it was produced mainly during stationary phase [1]. In this study, xanthan production in stationary phase was

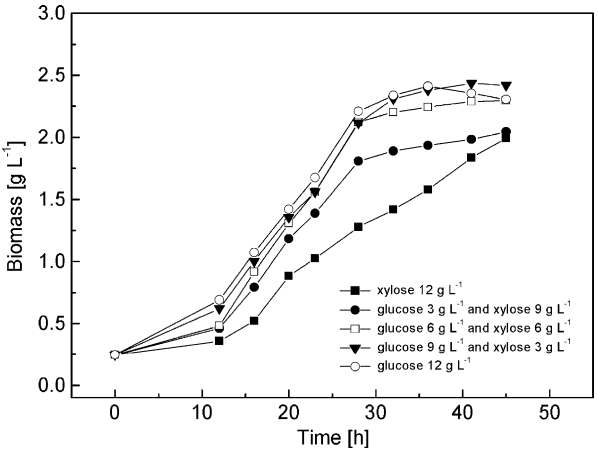

Fig. 1 The time course of cell concentration of *Xanthomonas campestris* in glucose, xylose, and glucose/xylose mixture

investigated. After 36 h culture in glucose media (at the end of the growth phase), certain amount of sugar was added in the broth (20 g of glucose or mixture per liter broth) and cultured again.

Figure 2a describe xanthan fermentation on the glucose media. It could be seen that the generation of xanthan ceased at about 40 h when the final concentration of xanthan was 18.20 g L^{-1}. The fermentations on mixed substrates were shown in Fig. 2b, c, and d. It was found that glucose and xylose were utilized simultaneously. In the media containing 75% glucose/25% xylose, the glucose was used up after 38 h of fermentation (Fig. 2b); in the 50% glucose/50% xylose fermentation (Fig. 2c), the glucose was completely consumed after 29 h, and in the 25% glucose/75% xylose fermentation (Fig. 2d), the glucose was used up after about 22 h. On the other hand, xylose was metabolized stably in all of the three mixed substrates and compared with glucose, contribution of xylose to xanthan production could be ignored (Table 1).

A summary of fermentation parameters was shown in Table 1. when the glucose decreased from 100% to 25%, glucose consumption rate, xanthan production rate and the yield of xanthan to total sugar decreased from 0.44 g L^{-1} h^{-1} to 0.25 g L^{-1} h^{-1}, 0.21 g L^{-1} h^{-1} to 0.04 g L^{-1} h^{-1}, and 0.45 g g^{-1} to 0.09 g g^{-1}, respectively, while the yield to glucose increased from 0.45 g g^{-1} to 0.61 g g^{-1}, when its fraction decreased from 100% to 50%. On the other hand, the xylose consumption rate remained constant (about 0.053–0.060 g L^{-1} h^{-1}) and only 2.5–2.9 g L^{-1} xylose was utilized after 48 h culture whatever the ratio of glucose and xylose was.

It was known that both glucose and xylose could be utilized by *X. campestris*. Figure 2 and Table 1 showed that the two sugars were metabolized simultaneously but the ability of *X. campestris* utilizing the two sugars were significantly different. Glucose consumption rate varied with the change of its fraction while xylose consumption rate was low and independent with its fraction, so the reason of the variety of xanthan production rate was mainly because of the glucose concentration at the fermentation start up. The Table 1 also showed that the yield of xanthan to glucose was not increased with the decrease of glucose fraction all along. When the ratio of glucose to xylose was 1:1, the yield of xanthan to glucose was highest. When the fraction of glucose was 25%, the yield to glucose was much lower (0.33 g g^{-1}). The results were caused by the low concentration of glucose and the

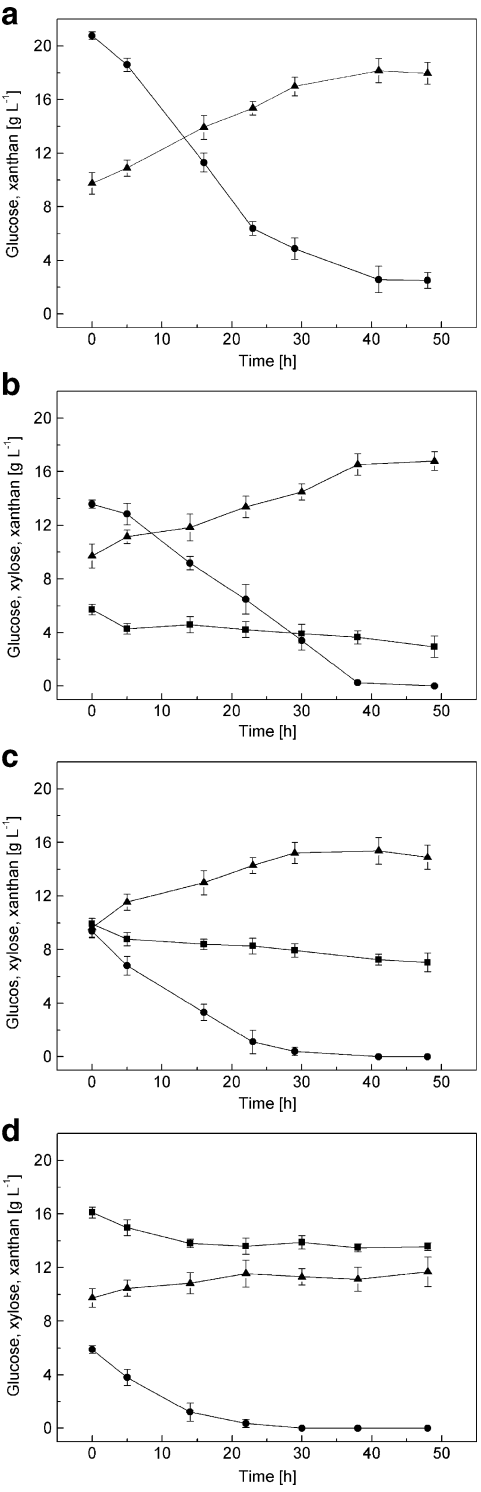

Fig. 2 The time course of glucose, xylose, and xanthan concentration during the production phase of xanthan fermentation with glucose media (20 g L^{-1} glucose, **a**), 75% glucose/25% xylose mixture (15 g L^{-1} glucose and 5 g L^{-1} xylose, **b**), 50% glucose/50% xylose mixture (10 g L^{-1} glucose and 10 g L^{-1} xylose, **c**) and 25% glucose/75% xylose mixture (5 g L^{-1} glucose and 15 g L^{-1} xylose, **d**). Glucose (*filled circle*), xylose (*filled square*), xanthan (*filled triangle*)

Table 1 Xanthan fermentation parameters in production phase on different ratios of glucose and xylose.

	Proportions of glucose and xylose in mixture			
	100% glucose	75%glucose/ 25%xylose	50% glucose/ 50% xylose	25% glucose/ 75% xylose
Maximum xanthan concentration (g L^{-1})	18.16	16.78	15.38	11.69
Yield of xanthan to glucose in stationary phase (g g^{-1})	0.45	0.52	0.61	0.33
Yield of xanthan to total sugar in stationary phase (g g^{-1})	0.45	0.37	0.30	0.09
Xanthan production rate (g L^{-1} h^{-1})	0.21	0.18	0.14	0.04
Glucose consumption rate (g L^{-1} h^{-1})	0.44	0.35	0.31	0.25
Xylose consumption rate (g L^{-1} h^{-1})	–	0.057	0.060	0.053

inhibition of xylose on the utilization of glucose [13]. In real industrial applications, hydrolysate of lignocelluloses is a mixture of glucose and xylose in which xylose fraction is less than 50% because there is much cellulose than hemicellulose in lignocelluloses [26, 27] and the hemicellulose is easy to degrade by certain pretreatment [28, 29]. Hence, these results suggested that xylose could not be utilized efficiently by *X. campestris*. The concentration of glucose rather than the total sugar was the main factor to determine the xanthan production.

Characteristics of the Structures of Xanthan Derived from Different Glucose/Xylose Mixture

Generally, the molecule of xanthan has a main chain of β-1, 4-D-glucan cellulosic backbone; every alternate glucose residue has a side chain consisting of a glucuronic acid residue between two mannose units. The terminal mannose moiety is pyruvated at C-4 and C-6 while acetate groups are linked to C(6) position of internal mannose as substituents. The values of molecular weight and the level of substitutions as well as the fermentation process are all influenced by nutrition in medium or operational conditions [17–21]. Accordingly, in this study, the value of acetate and pyruvate contents was investigated by HPLC [23] and average molecular weight of xanthan was evaluated by comparing the intrinsic viscosity ([η]).

Figure 3 showed the acetate and pyruvate contents in xanthan derived from various proportions of two sugars. It was shown that both the acetate and pyruvate contents increased with the increase of xylose fraction. When the xylose fraction increased from 0% to 50%, the pyruvate and acetate content xanthan increased from 2.43% to 3.78% and 2.55% to 7.05%. Compared with the pyruvate content, the acetate content increased more significantly.

Figure 4 shows the intrinsic viscosity values of xanthan produced from the glucose media and glucose/xylose mixed media. It was seen that the value of [η] of xanthan produced from the glucose/xylose mixed media was higher than that from the glucose media. When the fraction of xylose was 10%, the value of [η] was maximal. Since there was a positive correlation between [η] and average molecular weight [24, 25], these results reflected the relationship between xanthan molecular weight and the proportion of two sugars in the mixture.

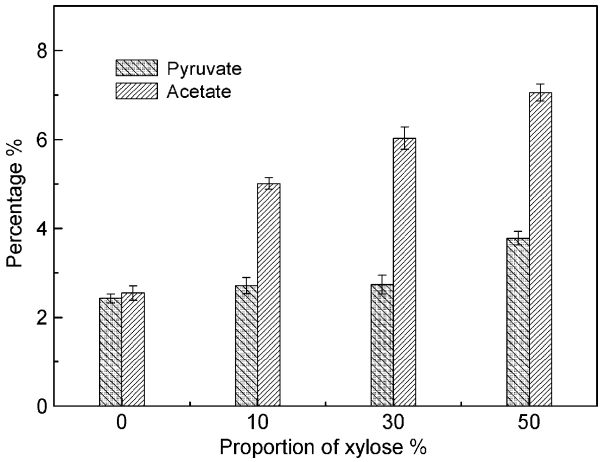

Fig. 3 Pyruvate and acetate contents in xanthan produced from various glucose/xylose mixtures

Figure 5 shows the rheological properties of xanthan derived from glucose media, mixture media, and commercial xanthan. It could be seen that when the shear rate increased from 22.4 s^{-1} to 109.6 s^{-1}, the apparent viscosity of xanthan derived from glucose media, 90% glucose/10% xylose media, 70% glucose/30% xylose media and 50% glucose/50% xylose media was decreased from 19.2, 21.9, 22.6, and 21.0 mPa·s to 10.8, 12.3, 12.1 and 11.0 mPa·s, respectively while the apparent viscosity of commercial xanthan was decreased greatly from 53.1 to 19.8 mPa·s.

Usually, the value of acetate and pyruvate content in xanthan produced by *X. campestris* was in variable amounts on the side chains. Karolyn reported that the two substitutes were 4.5% and 4.4%, respectively [30]; Casas found that they varied from 1.53% to 4.44% and 1.43% to 1.97% respectively in different culture condition [18]. Lopez determined the commercial xanthan, and found the two substitutes were 4.83% and 3.74% [31]. In this study, it was found that the acetate and pyruvate content of xanthan produced in glucose/

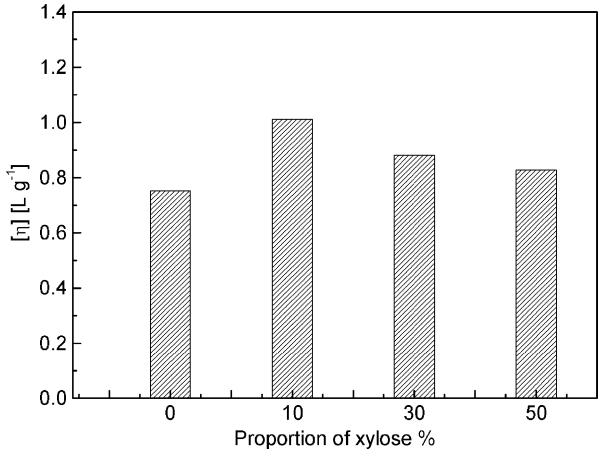

Fig. 4 Intrinsic viscosity of xanthan measured with 1 g L^{-1} xanthan gum in 0.1 g L^{-1} NaCl water solution at 25 °C

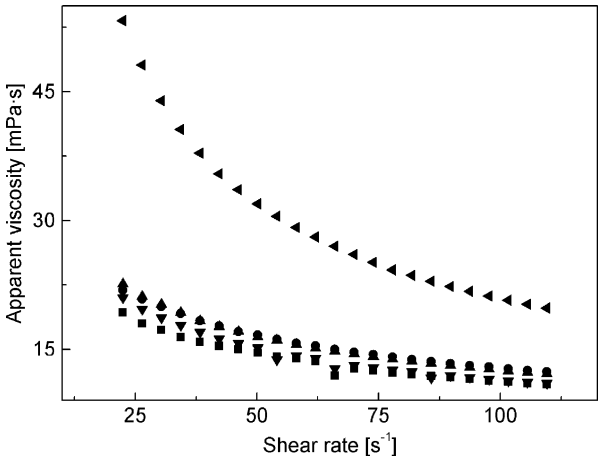

Fig. 5 Comparison of the apparent viscosity of xanthan derived from the media containing 100% glucose (*filled square*), 90%glucose/10%xylose (*filled inverted triangle*), 70%glucose/30%xylose (*filled triangle*), 50%glucose/50%xylsoe (*filled circle*), and commercial xanthan (*black left-pointing pointer*) in different shear stress

xylose mixture were all higher than that produced in glucose media, especially the acetate content, which was even higher than that of common commercial xanthan and previous reports. Molecular weight of xanthan is also an important factor for the rheological properties of xanthan solution. As it was difficult to determine the molecular weight of xanthan accurately [1], there were many different reports about it. Some author considered it was between 4 and 12×10^6 g mol^{-1} [1]; Wang [32] reported that the values was 2.5×10^6 g mol^{-1}; Milas [33] ever investigated the rheological properties using a kind of xanthan with a molecular weight of 7×10^6 g mol^{-1}. In our study, the results of intrinsic viscosity of xanthan derived from various media reflected the relationship between xanthan molecular weight and the proportion of two sugars in the mixture. It was found that all the molecular weights of xanthan derived from glucose/xylose media were higher than those of xanthan derived from glucose media. The biggest molecular was obtained when the media contained 10% xylose (Fig. 5). Using the parameter reported by Milas [33] ($K=1.7\times10^{-4}$, $\alpha=1.14$), the average molecular weight was 0.9×10^6 g mol^{-1}. This value was lower than previous reports. Because of the change of the structure (acetate content, pyruvate content, and molecular weight), xanthan derived from glucose/xylose media showed different rheological properties. Just as shown in Fig. 5, their viscosity all higher than that of xanthan derived from glucose media. It was also found that the viscosity of xanthan prepared by us was lower than that of commercial xanthan. This result consisted with the molecular weight determination and may be because of the bacterial properties.

These results suggested that xylose in glucose/xylose mixed media, was helpful to improve the quality of xanthan.

Conclusion

In this study, some fermentation parameters and structure characteristics of xanthan produced by *X. campestris* with different ratios of glucose/xylose mixture was investigated. Glucose was the preferred carbon source to produce xanthan while xylose could be utilized

at a very low consumption rate. With the decrease of the fraction of glucose, glucose consumption rate and xanthan production rate reduced while xylose was consumed stably. At the same time xylose enhanced the contents of acetate and pyruvate of xanthan and led to higher average molecular weight.

These results indicated that xylose was not efficiently utilized by *X. campestris* to produce xanthan. The concentration of glucose rather than the total sugar was the main factor to determine the xanthan production. But xylose was helpful to improve the quality of xanthan. The conclusion was useful for development of new processes for xanthan production from lignocellulosic materials.

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