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Effect of addition of sodium alginate on bacterial cellulose production by *Acetobacter xylinum*

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Abstract Bacterial cellulose (BC) production by *Acetobacter xylinum* NUST4.1 was carried out in the shake flask and in a stirred-tank reactor by means of adding sodium alginate (NaAlg) into the medium. When 0.04% (w/v) NaAlg was added in the shake flask, BC production reached 6.0 g/l and the terminal yield of the cellulose was 27% of the total sugar initially added, compared with 3.7 g/l and 24% in the control, respectively. The variation between replicates in all determinations was less than 5%. During the cultivation in the stirred-tank reactor, the addition of NaAlg changed the morphology of cellulose from the irregular clumps and fibrous masses entangled in the internals to discrete masses dispersing into the broth, which indicates that NaAlg hinders formation of large clumps of BC, and enhances cellulose yield. Because the structure of cellulose is changed depending on the culture condition such as additives, structural characteristics of BC produced in the NaAlg-free and NaAlg medium are compared using scanning electron microscopy (SEM), fourier transform infrared spectroscopy (FT-IR), X-ray diffraction (XRD). SEM photographs show some differences in reticulated structures and ribbon width and FT-IR spectra indicate that there is the hydrogen bonding interaction between BC and NaAlg, then X-ray diffraction (XRD) analysis reveals that BC produced with NaAlg-added has a lower crystallinity and a smaller crystalline size. The results show that enhanced yields and modification of cellulose structure occur in the presence of NaAlg.

Keywords Bacterial cellulose · *Acetobacter xylinum* · Sodium alginate · Stirred-tank reactor · Structure

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Introduction

Bacterial cellulose (BC), which is produced by some strains of *Acetobacter*, possesses particular physicochemical properties different from those of plant cellulose [21]. The unique properties of bacterial cellulose including high purity, high crystallinity, high mechanical strength, high water-holding capacity, good biocompatibility and high porosity have made BC a very potentially important industrial and biomedical material [1].

Acetobacter xylinum is a respective BC producer and its insoluble BC aggregates in different shapes. In addition, BC yield, structure and properties are different depending on the cultivation methods such as stationary culture and agitated culture in different kinds of reactors [20]. Although the static culture method for BC production is moderately successful on a small scale and it has been applied for production of some successful commercial cellulose products such as nata de coco, transducer diaphragms and wound care dressing materials, it is not applicable to the large-scale industrial production because of the long cultivation time, large surface areas and high labor force [14]. Agitated culture, on the other hand, is more suitable for the commercial-scale production as higher production rates can be achieved. So with the aim of improving BC production in agitated culture, the screening of suitable strains, improvement of the producing organisms, medium components and some basic operational parameters about different kinds of

reactors, including optimal oxygen supply, volumetric agitation power and so on, have been investigated [7, 11–13]. But this method has posed some problems, e.g. spontaneous appearance of cellulose non-producing mutants and serious clump forming problem, which result in a decline in cellulose yield and non-uniform structure and properties of BC.

Recently there are a few reports stating that addition of water-soluble polymers such as xanthan, agar, polyacrylamide-co-acylic acid (PCA), and acetan can increase relative viscosity of the broth to reduce shear stress, hinder coagulation of BC during the cultivation to form uniform smaller pellets, which are advantageous to transfer nutrients and oxygen into bacterial cells located inside and on the surface of the cellulose matrix, and further promote BC productivity into the medium, will assist in enhancing BC productivity [1, 3, 9, 10]. On the other hand, various water-soluble polymers added into the medium are known to interfere with the aggregation of microfibrils into a normal ribbon assembly, affect the crystallization of cellulose I_{α} and I_{β} and introduce the characteristic properties of these additives to attain BC composite materials [6].

Considering that sodium alginate is also a water-soluble polysaccharide and there are many –COOH and –OH groups, NaAlg has some effects on cellulose production and the modification of the cellulose occurs during microbiological synthesis by introducing NaAlg into the medium [17].

The present research aimed to systematically studying the effects of NaAlg on BC production by *Acetobacter xylinum* NUST4.1 and modification of cellulose structure. Firstly, we have made investigations to see that NaAlg can change BC morphology to solve the serious clump forming in the stirred-tank reactor and can improve BC production. Secondly, some characteristic structural analyses of these BC samples synthesized in the presence and absence of NaAlg have been determined by SEM, FT-IR, XRD.

Materials and methods

Bacterial strains

Acetobacter xylinum NUST4.1 in this study was isolated from the natural sources as a high BC producer.

Medium and cultivation

The seed medium contained (w/v): glucose 2%, solid corn steep liquor 0.6%, $(NH_4)_2SO_4$ 0.6%, KH_2PO_4 0.1%, $MgSO_4{\cdot}7H_2O$ 0.04%, initial pH value 6.0. The culture medium for BC production contained (w/v): glucose 1.8%, sucrose 2.1%, solid corn steep liquor 2.0%, $(NH_4)_2SO_4$ 0.4%, KH_2PO_4 0.2%, $MgSO_4{\cdot}7H_2O$ 0.04%, initial pH value 6.0. NaAlg (China Medicine (Group) Shanghai Chemical Reagent Co., China) was of chemical grade and used in various concentrations. In a stirred-tank reactor, 0.02% (v/v) anti-foam agent was added when necessary.

One milliliter of the cell suspension stored at −20°C was added to 100 ml of the feed medium in a 500-ml conical flask and shaken at 200 rpm and 29°C for 12 h in a rotary shaker (SHA-C, GUOHUA Co., Changzhou, Jiangsu, China). The resulting suspension was introduced into 250-ml conical flasks containing 45 ml of culture medium at a level of 10% (v/v). Cultivations were carried out at 29°C and 150 rpm for 5 days in a rotary shaker (SHZ-82, GUOHUA Co., Changzhou, Jiangsu, China) in triplicate experiments. Cultures in L1523 stirred-tank reactor (13 l nominal volume, 7 l working volume, Bioengineering AG, CH 8636 Wald, Switzerland) were carried out for the cultivation of *A. xylinum* NUST4.1 at 200 rpm and 29°C for 72 h. The preculture was prepared as described above. The airflow rate was supplied at 3 l/min.

Analytical methods

The sample clumps produced in shake flask were taken from the medium directly and washed with tap water. The samples' supernatant was saved for the determination of the total sugar concentration. The samples produced in the stirred-tank reactor were centrifuged at 10G (TGL-16G, ANTING Co., China) for 10 min, then the precipitate was washed with tap water, the supernatant from the first centrifugation was saved for the determination of the total sugar concentration. The clumps harvested from shake flasks and precipitate centrifuged from stirred-tank reactor were treated with 4% (w/v) NaOH at 80°C for 2 h to solubilize impurities and cells attached to cellulose and thoroughly washed in tap water until the pH of water became neutral. The purified BC was dried to constant weight at 80°C and weighed.

The total sugar concentration in the samples' supernatant was analyzed using the modified dinitrosalicylic acid (DNS) method [22].

The culture broth was suspended in 0.2% (w/v) cellulase in acetic acid–sodium acetate buffer (pH 5.0) and hydrolyzed for 2 h at 50°C, then filtered through filter paper. Cell growth was evaluated by measuring the absorbance of the filtrate at 600 nm.

The purified BC samples as described above were coated with gold. Scanning electron microscope observations were performed using JEOL JSM-6380 LV at 15 kV.

A Bruker EQUINOX55 remote sensing fourier transform infrared spectroscopy was used to collect transmission spectra with KBr wafer. N–O′KI crystalline index was calculated as N–O′KI = $\alpha_{1,372}/\alpha_{2,900}$ where $\alpha_{1,372}$ was IR band intensity in 1,372 cm^{-1} (CH bending vibration' frequency)

and $\alpha_{2,900}$ was IR band intensity in 2,900 cm^{-1} (CH and CH_2 stretching vibration' frequency) [19]. Estimation of the I_α fraction was carried out using the integrated intensities of the absorbance peaks at 750 and 710 cm^{-1} that were respectively, characteristic of I_α and I_β allomorphs. Assuming that the absorption at the above two peaks are proportional of the mass of I_α and I_β allomorphs, the I_α fraction was written as $f_\alpha = 2.55 \times A_{750}/(A_{750} + A_{710}) - 0.32$ [4].

X-ray diffraction spectra were recorded using a Bruker D8 ADVANCE X-ray powder diffractometer at 40 kV and 30 mA (Cu$K\alpha$ radiation) to analyze BC crystalline structure and crystal size. Scans were performed over the 5–40° 2θ range using step 0.1° width. The crystallinity index was calculated as Cr I = $(I_{002} - I_{am})/I_{002}$, where, I_{002} is the overall intensity of the peak at 2θ about 22° and I_{am} is the intensity of the baseline at 2θ about 18° [15]. The crystallite size was calculated by the Scherrer equation [23].

Results

Effect of addition of NaAlg on BC production in shake flasks

Shake flask experiments were performed without NaAlg to determine how the bacteria would behave in the agitated culture. The optimum culture medium had been determined through homogenous design and monofactorial experiment (results not shown), which could promote cell growth and BC production in comparison with the HS medium [5]. Cellulose produced in the shake flasks occurred as big oval clumps in a clear medium. BC production by *A.xylinum* NUST4.1 in the medium containing NaAlg in the range of 0–0.1% (w/v) was shown in Fig. 1. BC production by *A.xylinum* NUST4.1 without NaAlg was 3.7 g/l, while BC production was the highest, 6.0 g/l, at 0.04% NaAlg. However, there is no enhancing trend toward higher BC production with increasing NaAlg concentration, which suggests that NaAlg with higher concentration may do harm to BC production due to an increase of broth viscosity.

The time courses of cellulose production, cell and total sugar concentration in shake flasks are shown in Fig. 2a, b. In the absence of NaAlg, cells grew exponentially after a 24 h lag period and linearly between 48 and 72 h of cultivation time, then reached the stationary phase. But the addition of 0.04% NaAlg can significantly shorten the lag period and accelerate cell growth in the early phase of culture. Furthermore, when cell reached the logarithm period, cellulose also began to form and increase all along due to continuous cell growth regardless of NaAlg and NaAlg-free in the medium. Total sugar consumption with or without NaAlg was similar. However, total sugar consumption by *A.xylinum* NUST4.1 in the 0.04% NaAlg-added medium

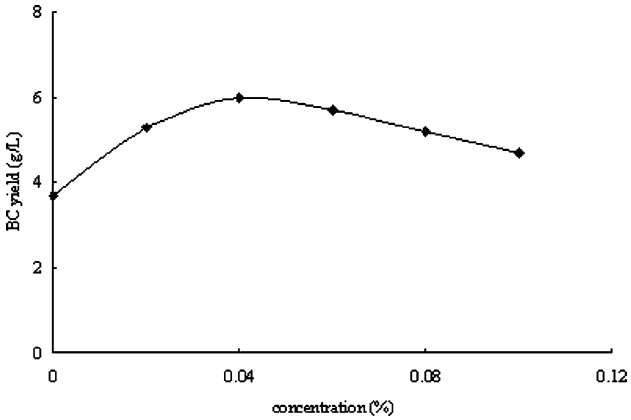

Fig. 1 The effect of NaAlg concentration of BC production by *A. xylinum* NUST4.1 in the shake flask

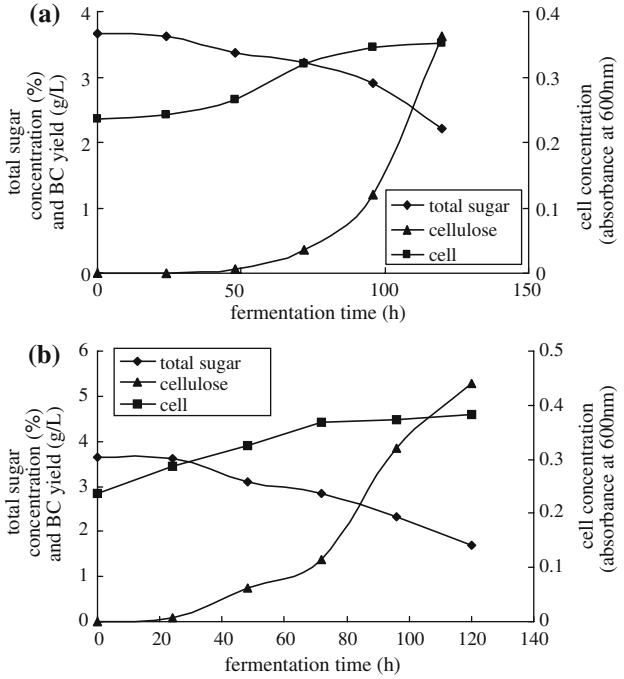

Fig. 2 Dynamics of cellulose synthesis by *A. xylinum* NUST4.1 in the shake flask. **a** 0% and **b** 0.04% NaAlg

was 2.0%, which is higher than 1.5% in the control medium without NaAlg. In addition, the terminal yield of the cellulose was 27% of the total sugar initially added in the presence of 0.04% NaAlg, compared with 24% in the control. Therefore, the addition of 0.04% NaAlg promoted cell growth and enhanced BC production in the shake flask.

Effect of NaAlg on BC production in a stirred-tank reactor

In a stirred-tank reactor, BC production by *A. xylinum* NUST4.1 was performed without NaAlg and with 0.04% NaAlg. Without NaAlg, cellulose was produced with irregular

clumps of different sizes and fibrous shapes, which was entangled in the internals such as in the impeller, on the sampling tube, on the pH electrode, dissolved oxygen probe, thermocouple. This resulted in non-uniform sampling and weak reproducibility of measurement during the culture courses. However, it's surprising that the problems of clumping did not occur at 0.04% NaAlg. During the course of inoculation, the suspension became gradually viscous due to BC production. The changes of cell and total sugar concentration and BC production in the stirred-tank reactor during the culture period were similar to those in the shake flask (Fig. 3). The maximum yield reached 1.89 g/l for 60 h at the NaAlg concentration of 0.04%, which is 1.7-fold higher than 1.09 g/l in the control medium without NaAlg. However, BC production by *A. xylinum* NUST4.1 in the stirred-tank reactor was much lower than that in the shake flask. The reason was apparent that culture broth in the stirred-tank reactor was more viscose than that in the shake flask, which resulted in the inhomogeneity of dissolve oxygen and nutrition transfer in the culture broth.

BC structure

The morphological structures of BC obtained with and without addition of NaAlg were analyzed by SEM, presented in Fig. 4a, b. The results show that these samples consist of ultrafine fibrils, which form the reticulated structure. But careful observations of the photographs reveal some obvious differences in surface structures and ribbons width of both types of BC samples. BC produced in the shake flask in the control medium without NaAlg was characterized by more compact and highly extended structure than that in the NaAlg-added medium. In contrast, BC produced in the NaAlg-added culture contained nets with many holes. In the surface of net there were many particles, which were probably composed of NaAlg. The inside one was composed of many ultrafine ribbons, which were curved and entangled with each other. The ribbons of BC

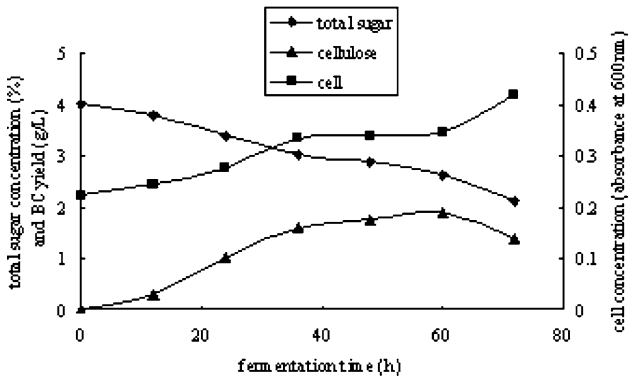

Fig. 3 Dynamics of cellulose synthesis by *A. xylinum* NUST4.1 in the stirred-tank reactor with 0.04% NaAlg

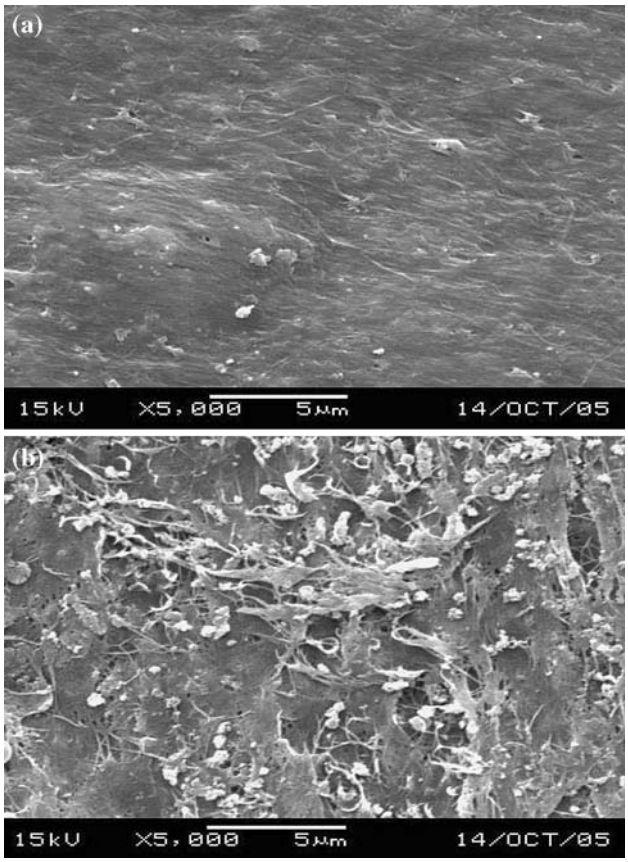

Fig. 4 Scanning electron micrographs of bacterial cellulose produced by *A. xylinum* NUST4.1in the medium containing NaAlg in the shake flask. **a** 0% and **b** 0.04% NaAlg

with NaAlg had a broader range of width than those without NaAlg, which would extend new applications of BC, because ribbon width influenced BC properties such as water-holding capacity, viscosity of wet BC suspension and mechanical properties.

Fourier transform infrared (FT-IR) spectroscopy of BC samples was carried out in order to detect any peak shift that could be attributed to weak interactions between the two polymers, such as hydrogen bonding or complexation. Figure 5a, b show the FT-IR spectra of BC produced in the presence and absence of NaAlg in the wavelength ranges of 3,500–500 cm^{-1}. The characteristic bands of cellulose synthesized in the control medium appeared at 3,231 cm^{-1} for hydroxyl groups stretching vibration, at 2,895 cm^{-1} for C–H stretching vibration, at 1,429 cm^{-1} for C–H bending vibration and at 1,059 cm^{-1} for C–O–C and C–O–H stretching vibration of sugar ring. Meanwhile, the spectrum of BC with NaAlg showed the peak at 3,170 cm^{-1} for hydroxyl groups stretching vibration. The absorption maxima of stretching vibration of hydroxyl bonding shifted toward lower wavenumbers and the hydroxyl stretching bands became much broader in the presence of NaAlg. Moreover, there was a significant difference in the region of

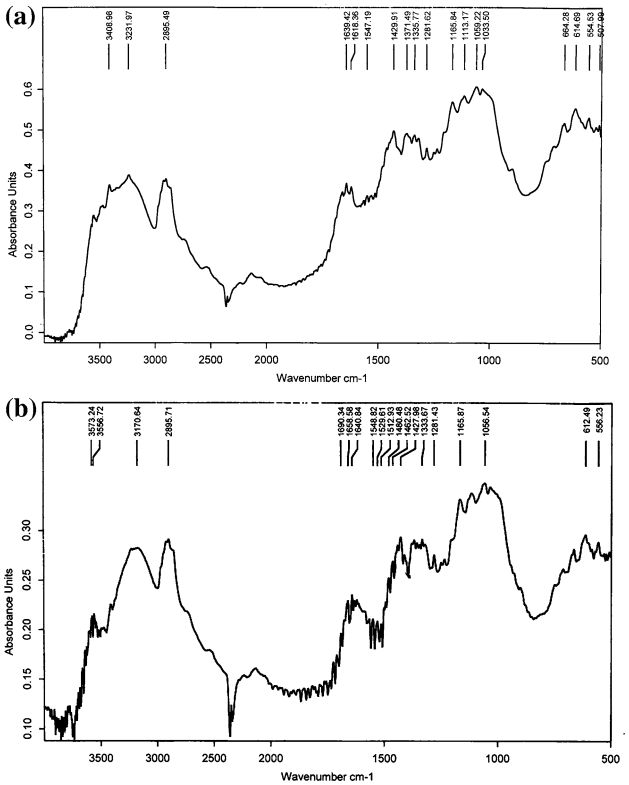

Fig. 5 FT-IR spectra of bacterial cellulose produced by *A. xylinum* NUST4.1 in the medium containing NaAlg in the shake flask. **a** 0% and **b** 0.04% NaAlg

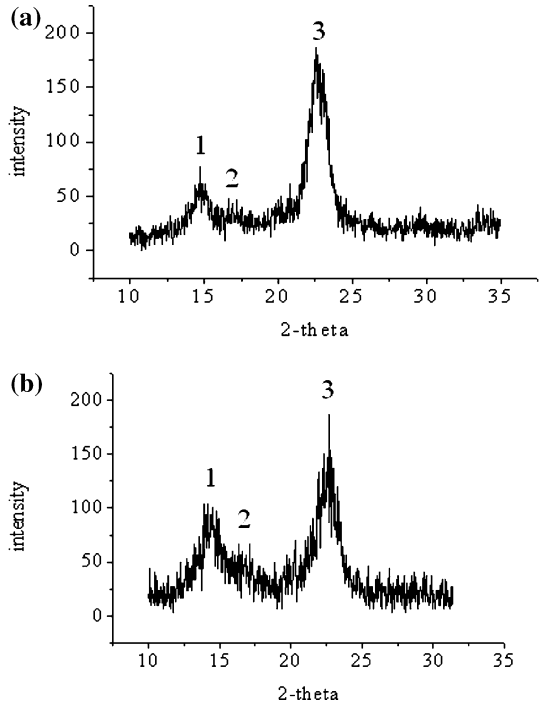

Fig. 6 X-ray diffraction patterns of bacterial cellulose produced by *A. xylinum* NUST4.1 in the medium containing NaAlg in the shake flask. **a** 0% and **b** 0.04% NaAlg

1,427–1,548 cm^{-1}. These absorption peaks were the asymmetric and symmetric –COO stretching vibrations. The change in the –COO band in the spectrum, suggests that there are hydrogen bonds between groups of BC and NaAlg resulting in strong interaction of inter-molecules. The fact strongly supports that BC can be modified by introducing NaAlg in the course of the biosynthetic environment.

X-ray diffraction is often used for structure measurement of polymers. The diffraction diagram indicated that BC synthesized in the NaAlg-free and NaAlg culture had three characteristic diffraction peaks standing for crystal plane ($\bar{i}$10), (110), (200), respectively, which revealed both of them were I crystal cellulose (Fig. 6a, b) [14]. But Fig. 6 also shows some differences in Bragg angles of peak 1 and peak 3 of both types of BC samples. The crystalline index of BC with NaAlg was lower than that of BC without NaAlg, which accorded with N-O′KI crystalline index calculated according to FT-IR spectra. And the crystallite size of (002) crystal plane was smaller too.

I_α fraction was respectively, 0.897 and 0.856 in the absence and presence of NaAlg, which displayed that two BC samples synthesized by *A. xylinum* NUST4.1 had abundant I_α allomorph, which was larger than the previous reports [8]. It was reported that the mass fraction of cellulose I_α was closely related to the crystallite size of microfibrils of BC. Addition of NaAlg can lead to the formation of crystallites of a smaller size. The results presented in Fig. 6 and Table 1 show that the addition of NaAlg can influence crystal structure of cellulose.

Discussion

Joseph et al. [10] reported that 7-day BC production by *A.xylinum* BPR2001 rose from 2.7 to 6.5 g/l at a shake speed of 175 rpm and from 1.7 to 3.7 g/l at shake speed of 375 rpm at PCA concentrations of 0–0.3%. In the shake flask experiments, a significant increase in BC production was also attained by the addition of NaAlg, a water-soluble polysaccharide in our research. The maximum BC yield by *A. xylinum* NUST4.1 was 6.0 g/l in the 0.04% NaAlg-based medium in the shake flask, which is 1.62-fold greater than 3.7 g/l in the control medium without NaAlg. Moreover, the cellulose yield was 27% of the total sugar initially added, which is higher than 24% in the control.

Although other polymeric additives such as xanthan, agar, acetan were investigated to hinder formation of large clumps of BC and enhance BC production in either jar fermentor or airlift reactor, there are remarkable differences in BC enhancing extent according to different strains, additives and additives concentration [1, 3, 9]. Bae et al. [1] reported that the maximum BC production of *A.xylinum* BPR2001 at 0.4% (w/v) agar was 12.8 g/l compared with

Table 1 Structural features of bacterial cellulose produced by *A. xylinum* NUST4.1 in the shake flask in the medium with or without 0.04% NaAlg-added

NaAlg concentration in the medium (%, w/v)	Structural features			
	N–O′KI crystalline index	Cellulose I_α (%)	Crystallinity (%)	Crystallite size of (002) (nm)
0	1.3053	89.7	78	5.8
0.04	0.7778	85.6	59	5.1

8 g/l without agar and the mutant EP1, generated from *A.xylinum* BPR2001 produced 11.6 g/l of BC at 0.6% agar, while only 5.5 g/l was produced in the control in a 10 l stirred-tank reactor. On the other hand, BC production of *A.xylinum* BPR2001 at 0.1% agar reached 8.7 g/l compared with 6.3 g/l in the control in a 50 l internal loop airlift reactor [3]. In our research, the addition of NaAlg influences not only the morphology of BC but also the cell growth and cellulose yield in the stirred-tank reactor. Actually, the addition of NaAlg to the reactor prevented clump formation and shortened lag period of cells. The maximum yield of BC reached 1.89 g/L in 60 h compared with 1.09 g/l in 72 h in the control.

The above results show that BC production by *A. xylinum* NUST4.1 in the shake flask was similar to that by *A. xylinum* BPR2001 and the BC yield by *A. xylinum* NUST4.1 was much lower than that *A. xylinum* BPR2001 and EP1 in the stirred-tank reactor. The main reason is that *A. xylinum* NUST4.1 is more instable than *A. xylinum* BPR2001 in the stirred-tank reactor, which should be further studied, though it is worth noting that the amount of NaAlg-adding was lower than that added by other workers, but its effect on BC production was obvious.

Hirai et al. [6] described the effects of different polymeric additives on the formation of microfibrils of BC. The effect of NaAlg on reticular structure, crystallinity and crystallite size and mass fraction of cellulose I_α of BC were examined by SEM, FT-IR, XRD. The ribbons produced in the NaAlg-added medium appeared broader range in width compared to the control as observed by SEM. Furthermore, NaAlg was wrapped in the network built of entangled cellulose ribbons. But BC produced in the control medium was denser. FT-IR analysis revealed there was the interaction from the shift of –OH and C–O–C bands in the presence of NaAlg. XRD analysis showed that the crystalline structure was different in the crystallinity and crystalline size. It suggests that BC produced by *A.xylinum* NUST4.1 can be modified by introducing NaAlg into the medium and can combine the properties of cellulose and NaAlg, e.g. water-absorption capacity, gas-penetrating ability, metal-ion chelateding capacity and separation ability of aqueous–organic mixtures and so on [2, 16–18].

Further research on determining the properties of the modified BC such as mechanical properties, thermal stability, gas- or liquid-penetrating capacity, metal-ion chelateding capacity is under way. In addition, some works are needed to thoroughly understand the underlying mechanism in the interaction of BC and NaAlg and the fundamental reasons of decreasing BC production by *A.xylinum* NUST4.1 in the stirred-tank reactor in comparison with in the shake flask. In conclusion, this work provides the kind of new polymeric additive, NaAlg, which enhances BC production and modifies cellulose structure.

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