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Characterization of Cellulose Production by a *Gluconacetobacter xylinus* Strain from Kombucha

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Abstract The aims of this work were to characterize and improve cellulose production by a *Gluconoacetobacter xylinus* strain isolated from Kombucha and determine the purity and some structural features of the cellulose from this strain. Cellulose yield in tea medium with both black tea and green tea and in Hestrin and Schramm (HS) medium under both static and agitated cultures was compared. In the tea medium, the highest cellulose yield was obtained with green tea (~0.20 g/L) rather than black tea (~0.14 g/L). Yield in HS was higher (~0.28 g/L) but did not differ between static and agitated incubation. ^{1}H-NMR and ^{13}C-NMR spectroscopy indicated that the cellulose is pure (free of acetan) and has high crystallinity, respectively. Cellulose yield was improved by changing the type and level of carbon and nitrogen source in the HS medium. A high yield of ~2.64 g/L was obtained with mannitol at 20 g/L and corn steep liquor at 40 g/L in combination. In the tea medium, tea at a level of 3 g/L gave the highest cellulose yield and the addition of 3 g/L of tea to the HS medium increased cellulose yield to 3.34 g/L. In conclusion, the *G. xylinus* strain from Kombucha had different cellulose-producing characteristics than previous strains isolated from fruit. Cellulose was produced in a pure form and showed high potential applicability. Our studies extensively characterized cellulose production from a *G. xylinus* strain from Kombucha for the first time, indicating both similarities and differences to strains from different sources.

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Introduction

Bacterial cellulose (BC), which is produced by some strains of the bacterial genera *Acetobacter, Agrobacterium, Gluconacetobacter, Rhizobium*, and *Sarcina*, represents a potential alternative to plant-derived cellulose for some specialist applications in the medical, acoustic, and other industries [12, 17]. Among the genera mentioned here, *Gluconacetobacter xylinus* (formerly *Acetobacter xylinum*) is one of the most commonly studied sources of BC because of its ability to produce relatively high levels of the polymer from a wide range of carbon and nitrogen sources in liquid culture [12].

The biochemical pathways of cellulose synthesis in *G. xylinus* are relatively well understood [12] and a large number of studies have been conducted with the aim of improving BC yield by this species for industrial application. A number of different strains of *G. xylinus*, such as BPR 2001 [14], E_{25} [9], and A9 [13], have been isolated from various sources (often fruit) and characterized with respect to cellulose production under different conditions. In particular, the role of different carbon and nitrogen sources as well as stationary and agitated cultures on BC production in these strains has been investigated. Individual strains have been found to perform better with respect

to BC production under some conditions than others. For example, *G. xylinus* BPR 2001 [17] and *G. xylinus* E_{25} [9] produce higher levels of BC under agitated and static cultures, respectively.

We recently isolated a *G. xylinus* strain from Kombucha, a popular fermented beverage originating in northeast China and which is prepared by fermentation of a tea broth supplemented with sugar by a coculture of bacterial (*G. xylinus, A. xylinoiides*, and *Bacterium gluconicum*) and yeast strains [3]. Almost all strains of *G. xylinus* previously characterized for cellulose production have been isolated from fresh fruit sources, and isolates from Kombucha have not been extensively characterized. Previously studied strains from fruit are unlikely to have been the subject of long-term subculture under static conditions, as is the case with strains from Kombucha. We hypothesized that such long-term culture in tea might have produced a strain that had different cellulose-producing characteristics than those typically encountered from other sources. We therefore undertook this study to both characterize and improve cellulose production for laboratory-scale experiments in a *G. xylinus* strain from Kombucha, which might have commercial potential for cellulose production.

Materials and Methods

Bacterial Strain, Culture Conditions, and Inoculum Preparation

The *G. xylinus* strain (K3) used in this study was previously isolated and identified in our laboratory from a commercially available Kombucha culture (Kombu Australia, Springwood, Queensland, Australia). It was maintained at −80°C on Protect Bacterial Preservers (Technical Service Consultants, Heywood, UK) and resuscitated by incubation on YPM (25 g/L mannitol, 5 g/L yeast extract, 3 g/L peptone, and 15 g/L agar) at 30°C for 2 days. Working cultures were routinely prepared on YPM and stored at 4°C until use.

The basic growth medium used for the strain was Hestrin and Schramm (HS) medium (20 g/L glucose, 5 g/L peptone, 5 g/L yeast extract, 2.7 g/L Na_2HPO_4, 1.15 g/L citric acid.H_2O, pH 5) [6]. A traditional tea-based medium (80 g/L sucrose and 3 g/L tea) was also used to culture the organism.

Inoculum was prepared by transferring a single colony from the YPM working culture plates into 100 mL of HS medium in 500-mL bottles and incubating the culture without agitation at 30°C for 2 days. The broth was shaken vigorously to release the attached cells from the cellulose pellicle and then aseptically filtered through sterilized gauze. The resulting cell suspension was used for all subsequent experiments. Experiments were performed by adding 10 mLl of inoculum into a 500-mL bottle containing 90 mL medium, which was then incubated without agitation (static) or with shaking at 60 oscillations per minute (agitated) at 30°C for 7 days.

Experimental Design

Cellulose yield of the *G. xylinus* strain in traditional tea medium under recommended conditions (with both green and black tea) was compared to yield under static and agitated culture conditions in HS. This was done to determine whether an artificial medium had the potential to improve cellulose yields from a strain adapted to growth in tea as well as to identify the most favorable of these incubation conditions for this strain.

Pellicle (gelatinous membranes of cellulose floating on the medium surface) and globules (irregular forms of cellulose in deep media) prepared from the HS cultures under static and agitated incubation conditions, respectively, were analyzed by nuclear magnetic resonance (NMR) spectroscopy to investigate purity and structural properties of the cellulose produced by this strain.

Cellulose yield by the strain in HS with one of six carbon or five nitrogen sources (Table 1) at both 10 g/L and 20 g/L was determined. Subsequently, five levels (10, 20, 30, 40, and 50 g/L) of the best performing carbon and nitrogen source on cellulose yield were investigated. The yield of cellulose was also investigated in tea medium using two types of tea (black and green) at four levels (0, 1.5, 3.0, and 6.0 g/L).

Analytical Methods

Cellulose Yield

The cellulose (pellicle or globules) from the *G. xylinus* cultures was purified by treatment with 0.5 M NaOH at 90°C for 1 h to eliminate attached cells, followed by three washes with distilled water to remove medium components and other residues. The purified cellulose was dried at 105°C to constant weight and the mass was determined [13].

Nuclear Magnetic Resonance

In addition to producing cellulose, some strains of *G. xylinus* have been found to synthesize acetan, a water-soluble xanthanlike, complex anionic branched heteropolysaccharide that might affect the purity as well as the yield of cellulose [7]. The presence of acetan, which can be produced by *G. xylinus* K3, was examined using solution-state ^{1}H-NMR spectroscopy. Samples were prepared following a previously described method [7]. Briefly, after *G. xylinus* growth (30°C for 7 days) spent HS was centrifuged, the

Table 1 The effect of carbon (20 g/L) and nitrogen (10 g/L) sources on cellulose production by *G. xylinus* under static culture conditions

Carbon source[a]	Cellulose yield (g/L)	Nitrogen source[b]	Cellulose yield (g/L)
Mannitol	0.82 ± 0.03	Corn steep liquor	1.07 ± 0.02
Fructose	0.28 ± 0.02	Peptone	0.85 ± 0.10
Glucose	0.28 ± 0.01	Yeast extract	0.85 ± 0.05
Sucrose	0.21 ± 0.01	Beef extract	0.75 ± 0.06
Maltose	0.15 ± 0.01	Malt extract	0.26 ± 0.04
Lactose	0.07 ± 0.00		

[a] These carbon sources were used by themselves to replace glucose in HS medium

[b] These nitrogen sources were used by themselves to replace peptone and yeast extract in HS medium

supernatant was precipitated with two volumes of ethanol. The precipitate was dissolved in 0.1 M NaOH (20 mLl) and subsequently washed with dichloromethane. Ethanol was added to the aqueous phase and the resulting precipitate was filtered, air-dried, and dissolved in the NMR solvent (H_2O with 50 μL D_2O). The resulting solution was filtered into a 5-mm NMR tube. ^{1}H-NMR spectra were measured at 298 K at 500 MHz on a Bruker DRX500 spectrometer. Spectra were acquired with presaturation of the water peak with a 6.0-μs 90° pulse and 1.5-s recycle delay. A total of 128 scans were recorded for each spectrum. The presence of acetan was determined by comparison of the ^{1}H-NMR spectra with that of native acetan [1].

The supramolecular structure of cellulose was determined using solid-state ^{13}C-NMR spectroscopy. Prior to loading, cellulose samples from *G. xylinus* cultures were rinsed with water and air-dried. NMR spectra were acquired at a ^{13}C frequency of 75.46 MHz and at 303 K on a Bruker MSL-300 spectrometer. The spectral width was 38 kHz, with a contact time of 1 ms. The rotor was spun at 5–6 kHz at the magic angle of 54.7° and experimental recycle times were 3 s. At least 2400 scans were accumulated for each spectrum.

Statistical Analysis

All quantitative data, unless otherwise stated, are presented as the means of triplicates, with error represented by standard deviation. Differences between individual values were determined by using a two-sample t-test on Minitab 14 for Windows® with significance based on a level of 5% ($P < 0.05$).

Results and Discussion

Cellulose-Producing Ability of the Strain

The *G. xylinus* strain investigated in this study gave significantly ($P < 0.05$) higher cellulose yields in the HS medium (~0.28 ± 0.02 g/L) than Kombucha medium with either green (0.20 ± 0.01 g/L) or black tea (0.14 ± 0.01 g/L) under static culture conditions. This demonstrates that the HS medium has potential to increase the amount of cellulose produced by this strain. Although the strain has been adapted to growth in tea in the long term as part of Kombucha culture, the higher levels of nutrients in the HS medium compared to the tea medium probably favored cellulose production in the former.

The cellulose yield of *G. xylinus* K3 in the static culture was not significantly higher than that in the agitated culture (0.26 ± 0.02 g/L) under the conditions of this study. We had speculated that this strain might be adapted to growth under static conditions by serial culture in Kombucha because, as indicated previously, cellulose production of particular strains of this species are often favored by either static or agitated conditions [9, 17]. This was not clearly the case in this study, but it is possible that the strain was adapted to growth in static culture in ways not investigated in our study. For example, lower cellulose yield in *G. xylinus* in serial agitated culture might occur due to genetic instability resulting in the gradual replacement of cellulose-producing cells by cellulose-nonproducing mutants [15].

Cellulose Purity and Structure

The purity of the cellulose was determined by the presence of acetan using solution-state ^{1}H-NMR spectroscopy. Comparison of ^{1}H-NMR spectra for both static and agitated samples to those reported in the literature [1] reveals that signals assigned to acetan were not observed, indicating that no detectable acetan was present in either sample. The absence of acetan production by a strain is advantageous in terms of cellulose production, because the cellulose is both purer and more nutrient energy is available for the production of cellulose.

Spectra obtained using solid-state CP/MAS ^{13}C-NMR spectroscopy for both the static and agitated cellulose samples are shown in Fig. 1. A comparision of these spectra to that of native cellulose [8] indicates that all observed

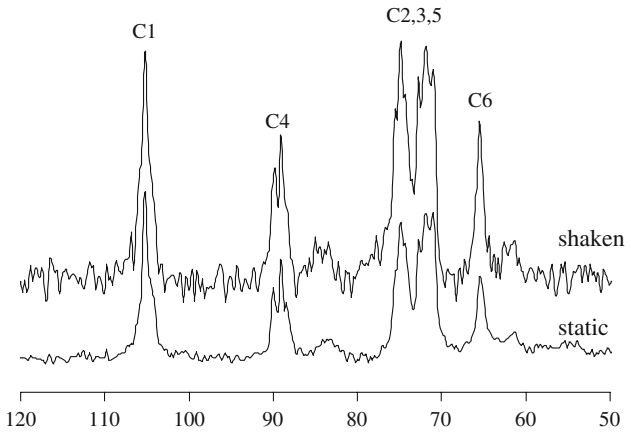

Fig. 1 ^{13}C-NMR spectra of cellulose samples from both static culture and shaken culture of *G. xylinus* after 7-day growth in HS medium

signals could be assigned to cellulose, confirming the relatively low levels of any other extracellular (solidlike) polymer. Bacterial cellulose from static culture showed a higher crystallinity index (80%) and content of cellulose I_α (65%) than that from agitated culture (76% and 50%, respectively). Previous studies reported that the effect of turbulent flow on the crystallization process of bacterial cellulose in the agitated culture might lead to the formation of crystallites of small size, which is closely related the mass fraction of cellulose I_β. As a result, the content of I_α in, and the crystallinity of, cellulose from the agitated culture was commonly lower than that of static culture cellulose [2, 16].

Improvement of Cellulose Production

Of the substrate sources tested (Table 1) the highest level of cellulose production was obtained when mannitol and corn steep liquor (CSL) were used as the sole source of carbon and nitrogen, respectively. It is well established that the preferential substrate sources for the production of cellulose by *G. xylinus* vary depending on specific strains. For example, the highest yield of cellulose is produced by *G. xylinus* BPR2001 in a medium containing fructose and CSL [10], whereas *G. xylinus* A9 produces its highest yield in a medium containing glucose and yeast extract [13]. Our study indicates that *G. xylinus* K3 has different prefered substrate sources for the production of cellulose than some other strains reported in the literature. Increasing the levels of mannitol up to 20 g/L or of CSL up to g/L enhanced cellulose production (2.64 ± 0.1 g/L), but cellulose yields decreased when higher concentrations of mannitol and CSL were used (Fig. 2). An optimal concentration of substrates for cellulose production has also been reported in other studies [11].

Studies on the effect of levels of green tea on cellulose yield in the traditional tea medium found that tea at a level of

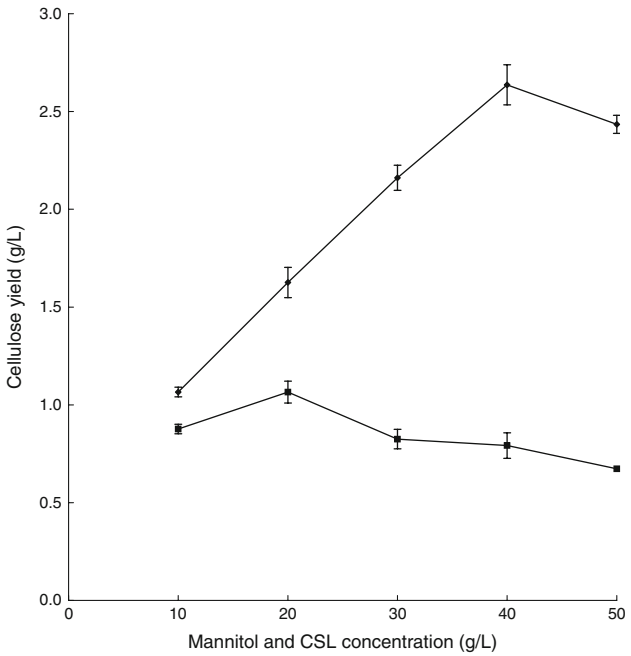

Fig. 2 The effect of mannitol (■) and CSL (◆) concentration on cellulose production by *G. xylinus* under static culture conditions

3 g/L gives the highest cellulose yield of 0.20 ± 0.02 g/L after 7 days of incubation, whereas at 6 g/L, all cellulose production was completely inhibited. The addition of a level of 3 g/L of green tea to the HS medium containing mannitol at 20 g/L and CSL at 40 g/L resulted in an increase in cellulose yield from 2.64 ± 0.10 to 3.34 ± 0.27 g/L. Positive effects of tea on bacterial cellulose production have previously been reported [4]. In particular, tea infusions (caffeine and theophylline) function as stimulators of cellulose production by preventing c-di-GMP (the most important factor in cellulose synthesis) from being destroyed by the enzyme phosphodiesterases. However, polyphenols in tea have antibacterial activity against a wide range of bacteria, such as *Staphylococcus aureus, Geobacillus stearothermophilus,* and *Pseudomonas* spp. [5]. Tea at high levels might therefore inhibit growth of *G. xylinus* and might be the reason why there was no cellulose produced when tea levels were 6 g/L.

In conclusion, our *G. xylinus* strain showed specific and independent cellulose-producing characteristics that were different from previously described strains. Few of these, however, were obviously related to its source of origin, as originally speculated. This strain is suitable for growth under static culture and its cellulose has high potential applicability as it has high crystallinity and purity. Cellulose yield can be improved by a number of strategies, among the most interesting being the inclusion of tea components in the medium. Further investigations into the effect of tea on the cellulose produced are required before this approach to increasing yield could be applied.

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