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Short communication

An alternative carbon source from konjac powder for enhancing production of bacterial cellulose in static cultures by a model strain *Acetobacter aceti* subsp. *xylinus* ATCC 23770

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Abstract

A new carbon source for bacterial cellulose production was successfully developed from konjac powder by using dilute acid hydrolysis and was detoxified by different alkaline treatment methods to remove microbial growth inhibitors. The various treatments included the addition of calcium hydroxide or sodium hydroxide to pH 10, and subsequent adjustment of the pH to 5 with acid as well as treatment with activated charcoal or laccase, respectively. The results showed that the detoxification effect using $Ca(OH)_2$ was much better than that using NaOH. If activated charcoal or laccase was added in the process, the detoxification effects would go further and bacterial cellulose production could be improved more. Based on the same concentration of total sugars, bacterial cellulose production using the hydrolyzates was three times higher than that using glucose, six times higher than that using mannose, and five times higher than that using glucose–mannose mixture as carbon source in static cultures. The addition of extra calcium in glucose media in the form of $CaCl_2$ at pH 5 did result in an improvement of less than 50% in BC production, which was not comparable to the $Ca(OH)_2$ treatments at pH 10. The possible mechanisms behind the findings were discussed and potential stimulatory factors for the fermenting bacterium formed during the alkaline processing deserve further attention. The results indicate that konjac powder could serve as a feedstock for bacterial cellulose production and cultivation of *Amorphophallus rivieri Durieu* would bring more economic benefits to farmers in future.

Keywords: *Acetobacter aceti*; Bacterial cellulose; Acid hydrolysis; Alkaline detoxification; Carbon source; Konjac powder hydrolyzate

1. Introduction

Compared with other natural plant cellulose, bacterial cellulose (BC) is one of the most promising biological based materials and a nano-biomaterial, which displays many unique properties including higher purity, higher crystallinity, higher degree of polymerization, higher water absorption and retaining capacity, higher tensile strength, and stronger biological adaptability (Backdahl et al., 2006; Iguchi, Yamanaka, & Budhiono, 2000; Klemm, Schumann, Udhardt, & Marsch, 2001; Klemm et al., 2006). This kind of material has broad prospective applications bringing tremendous economic and societal benefits in different fields, such as foods, textiles, paper, composite membranes, medicine, artificial skins and blood vessels, binding agents, loud speaker diaphragms and so on (Fontana et al., 1990,1997; Okiyama, Motoki, & Yamanaka, 1993; Shibazaki, Kuga, Onabe, & Usuda, 1993; Svensson et al., 2005; Wan et al., 2006). BC production has been demonstrated from glucose, sucrose, fructose, glycerol,

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mannitol and arabitol, among which mannitol and fructose are better carbon sources (Masaoka, Ohe, & Sakota, 1993; Oikawa, Morino, & Ameyama, 1995; Oikawa, Ohtori, & Ameyama, 1995; Ross, Mayer, & Benziman, 1991; Shoda & Sugano, 2005). The high economic cost of mannitol and fructose as well as relative low-yield production with these carbon sources limit industrial production and extended commercial applications of BC. Therefore, it is challenging and meaningful for us to look for a new approach to prepare a carbon source for high-yield BC production.

Amorphophallus rivieri Durieu is a perennial herbaceous plant, which grows in mountain or hilly areas in subtropical regions mainly in the Southeast Asia and Africa. Its tuber has been used as food and food additives in China and Japan for more than 1000 years (Zhang, Xie, & Gan, 2005). Konjac powder, produced from *A. rivieri Durieu* tuber, is one of the most economical and fruitful materials in China. Its main component is konjac glucomannan (KGM), which is a linear random copolymer of β-(1-4) linked D-glucose and D-mannose in the molar ratio of 1:1.6 with a low degree of acetyl groups (Nishinari, Williams, & Phillips, 1992; Smith & Srivastava, 1959). KGM can be hydrolyzed into monosaccharides including D-glucose and D-mannose by dilute acids. Actually, both sugars could be used as routine carbon sources separately for BC production in previous reports (Keshk & Sameshima, 2005). However, BC production using the konjac powder as a carbon source has not been investigated yet.

In this research a relatively low-cost carbon source of culture media was successfully developed from the cheap agricultural product konjac powder by using dilute acid hydrolysis and was satisfactorily detoxified by alkaline treatments to remove microbial growth inhibitors. The methods for hydrolysis and detoxification were simple and convenient. The yield of BC production was much higher than those using routine monosaccharides. Thus, the proposed new approach is an economical, practical and excellent way to prepare a new carbon source for mass production of BC in future.

2. Materials and methods

2.1. Microorganism and culture medium

Acetobacter aceti subsp. *xylinus* (previously *Acetobacter xylinum*) ATCC 23770 obtained from the American Type Culture Collection (ATCC, Manassas, VA) was used as a model strain and maintained on agar plates containing a seed culture medium (25 g/L D-mannitol, 5 g/L yeast extract and 3 g/L tryptone) and 20 g/L agar.

2.2. Hydrolysis of konjac powder

The konjac powder made from *A. rivieri Durieu* by Beilian Food Company (Shanghai, China) was hydrolyzed with sulphuric acid or hydrochloric acid. Firstly, konjac powder was soaked completely by dilute acids with a certain solid–liquid ratio and was agitated to mix. Then the hydrolysis was carried out in a 100 °C water bath. After hydrolysis, the hydrolyzate was harvested by filtration. Under the hydrolytic condition, sugar yield of konjac powder hydrolysis could reach 80% or more, and the concentration of total reducing sugars was about 20 g/L.

2.3. Detoxification of konjac powder hydrolyzate

The hydrolyzate of konjac powder contains a complex mixture of compounds that retard the growth of microorganisms. By referring to detoxification methods of lignocellulosic hydrolyzate (Jönsson, Pamqvist, Nivlvebrant, & Hahn-Hagerdal, 1998; Martinez, Rodriguez, York, Preston, & Ingram, 2000), several treatment methods for detoxification of konjac powder hydrolyzate were performed as shown in Table 1. Activated charcoal (Analytic grade, product no. 10006619) was purchased from Sinopharm

Table 1
Description of detoxification procedure in 12 different methods

Methods	Description of detoxification procedure
(1)	Adding NaOH to adjust pH value of hydrolyzate to 5.0
(2)	Adding NaOH to pH 5.0, and subsequently adding activated charcoal to 2%, then removing the activated charcoal and adjusting pH to 5.0 again
(3)	Adding NaOH to pH 10.0, and subsequently adding activated charcoal to 2%, then removing the activated charcoal and adjusting pH to 5.0 again
(4)	Adding NaOH to pH 10.0 and incubating at 30 °C for 12 h, and then adjusting pH value to 5.0 again
(5)	Adding NaOH to pH 10.0 and incubating at 30 °C for 12 h, and adjusting pH value to 5.0, then adding 2% activated charcoal, removing the activated charcoal and adjusting pH value to 5.0 again
(6)	Adding NaOH to pH 5.0, adding 5% laccase with 3.4 U/mL activity, incubating at 30 °C for 12 h, and adjusting pH value to 5.0 again
(7)	Adding $Ca(OH)_2$ to adjust pH value of hydrolyzate to 5.0
(8)	Adding $Ca(OH)_2$ to pH 5.0, adding activated charcoal to 2%, then removing the activated charcoal and adjusting pH value to 5.0 again
(9)	Adding $Ca(OH)_2$ to pH 10.0, adding activated charcoal to 2%, then removing the activated charcoal and adjusting pH value to 5.0 again
(10)	Adding $Ca(OH)_2$ to pH 10.0, incubating at 30 °C for 12 h, and adjusting pH value to 5.0 again
(11)	Adding $Ca(OH)_2$ to pH 10.0, incubating at 30 °C for 12 h, and adjusting pH value to 5.0, then adding activated charcoal to 2%, then removing the activated charcoal and adjusting pH value to 5.0 again
(12)	Adding $Ca(OH)_2$ to pH 5.0, adding 5% laccase with 3.4 U/mL activity, incubating at 30 °C for 12 h, and adjusting pH value to 5.0 again

Chemical Reagent Co., Ltd. (Shanghai, China) and laccase (product no. 53739) was obtained from Sigma–Aldrich (Steinheim, Germany).

2.4. Determination of reducing sugars

Concentrations of D-glucose, D-mannose in culture media and total reducing sugars in hydrolyzate of konjac powder were determined by using 3,5-dinitrosalicylic acid method (DNS method, Lindsay, 1973).

2.5. Seed culture and production of BC

Two loops of the strain were inoculated into a conical flask containing the seed culture medium mentioned above. The initial pH value of the medium was adjusted to 5.0 (Gherna, Pienta, & Cote, 1989) and was not regulated during flask cultures. The seed culture was incubated at 30 °C and 160 rpm on a rotary shaker for 1 day, and was then inoculated into a 100-mL liquid production medium by 6% capacity, which was cultivated at pH 5.0 (Gherna et al., 1989) and 30 °C in a static incubator for 8–23 days. The liquid production medium used for BC's production consisted of 5 g/L yeast extract, 3 g/L tryptone and the konjac powder hydrolyzate as a sole carbon source, in which the concentration of total reducing sugars was diluted to 15 g/L. For comparison, the konjac powder hydrolyzate was simply replaced by glucose, mannose or glucose–mannose mixture in other tests.

2.6. Harvest and weighing of BC

After cultivation, the bacterial cellulose membrane was washed successively with water, 0.1 mol/L NaOH solution in a boiling water bath for 30 min, and then washed by fresh water for three times to remove microbial product contaminants. The purified cellulose membrane was then collected by filtration on a quantitative filter paper (Xinhua Group Co., Ltd., Hangzhou, China) and dried to constant weight at 105 °C.

3. Results and discussion

3.1. Detoxification of konjac powder hydrolyzates and production of BC

Our previous study found the hydrolyzate of konjac powder contained many toxic compounds, which inhibited the growth of the bacterium *A. aceti* subsp. *xylinus* and subsequently no cellulose was produced. The konjac hydrolyzate was not capable to be used as a carbon source directly (data not shown). Therefore, it is necessary to have the konjac powder hydrolyzate detoxified before utilization.

It was reported that in the process of bioconversion of lignocelluloses to alcohol, $Ca(OH)_2$ and NaOH could be used for detoxification of hydrolyzate of lignocelluloses (Martinez et al., 2000). Therefore, 12 alkaline treatment methods were designed and carried out for the detoxification of hydrolyzates in this research. The methods included detoxification with calcium hydroxide or sodium hydroxide treatment, detoxification with alkaline treatments in combination with activated charcoal or laccase, as described in Table 1 of Section 2.3.

KGM is the main component of konjac powder, which can be hydrolyzed into a monosaccharide mixture composed of D-glucose and D-mannose in the molar ratio of 1:1.6. In order to investigate the effect of detoxification, a mixture of D-glucose and D-mannose (molar ratio 1:1.6) imitating the KGM hydrolyzate, D-glucose, D-mannose and the modified hydrolyzates were used as carbon sources, respectively, for comparison. In the research, all carbon source concentrations were adjusted to 15 g/L (determined by DNS method), and the volume of liquid media was 100 mL. The results are shown in Table 2.

Table 2 indicates that the detoxification effect with $Ca(OH)_2$ treatment was much better than that with NaOH treatment. BC membranes formed in the static cultures using the $Ca(OH)_2$-detoxified hydrolyzates after 8 days, but were not found in the cultures using the NaOH-detoxified hydrolyzates until the cultivations lasted to 23 day. The result also shows that the method (11) "Adjusting pH value of hydrolyzate to 10.0 by $Ca(OH)_2$, incubating at 30 °C for 12 h, and adjusting pH value to 5.0, then adding 2% activated charcoal into the hydrolyzate, then removing the activated charcoal and adjusting pH value to 5.0 again" was the most efficient way for detoxification, and the yield and productivity of BC were highest. The BC yield was about three times higher than that obtained with glucose, six times higher than that with mannose and five times higher than that with the KGM hydrolyzate imitator,

Table 2
Effects of different detoxification methods on BC production

Carbon source[a]	Production of bacterial cellulose (g)	
	Dry weight after 8 days	Dry weight after 23 days
D-Glucose	0.069 ± 0.013	–
D-Mannose	0.033 ± 0.013	–
Glucose–mannose (molar ratio 1:1.6)	0.041 ± 0.001	–
(1)	No membrane	0.077 ± 0.006
(2)	No membrane	0.164 ± 0.023
(3)	No membrane	0.134 ± 0.028
(4)	No membrane	0.098 ± 0.023
(5)	No membrane	0.130 ± 0.001
(6)	No membrane	0.163 ± 0.087
(7)	0.101 ± 0.004	–
(8)	0.123 ± 0.007	–
(9)	0.143 ± 0.023	–
(10)	0.172 ± 0.017	–
(11)	0.212 ± 0.047	–
(12)	0.113 ± 0.001	–

[a] The carbon source numbering from (1) to (12) were the hydrolyzates of konjac powder treated by different detoxification methods (see Section 2.3).

the glucose–mannose mixture (1:1.6). During 8-day cultivation the BC production order was (11) > (10) > (9) > (8) > (12) > (7) > glucose > glucose–mannose > mannose, in the comparison of BC yield.

The result showed that only $Ca(OH)_2$ treatment (method 7 and 10) was good enough for hydrolyzate detoxification since it gave 2.5- to 4-fold- higher BC yield compared with the glucose–mannose mixture (1:1.6). However, the detoxification effects would go further and BC production could be improved more if adding activated charcoal (method 8, 9 and 11) or laccase (method 12) in the processing. The result also showed that detoxification effects treated at pH 10 were much better than those treated at pH 5.0.

3.2. Effect of Ca^{2+} on BC production

Inorganic salts play an important role in BC production (Son et al., 2003). In this research, it was found that BC production using the $Ca(OH)_2$-treated hydrolyzates was much higher than that obtained with conventional carbon sources, glucose or mannose, and interestingly was even higher than that obtained with the KGM hydrolyzate imitator, the glucose–mannose mixture (1:1.6). It stood a good chance that Ca^{2+} was the key factor for this improvement. As glucose is a routine and good carbon source for BC production, it would be more readily understood if BC production would be improved after addition of calcium cation. Therefore, the effect of Ca^{2+} concentration on BC production in a glucose medium was investigated by addition of Ca_2Cl. The results are shown in Fig. 1.

Fig. 1 indicates that the addition of Ca^{2+} could obviously improve the BC production in the static culture under a certain concentration range ($\leqslant$7 mmol/L). The highest BC yield with the addition of 1 mmol/L Ca^{2+} was only 1.5 times higher than that without addition. The data also shows there was no big difference in BC production in the calcium concentration range of 1–7 mmol/L. However, the production declined immediately if the concentration exceeded 10 mmol/L, and BC would not be produced any more if the concentration exceeded 50 mmol/L. This result certified that Ca^{2+} was one of important factors in BC production improvement, but it seems not a sole factor since the improvement in BC yield was not comparable to that with $Ca(OH)_2$ treatments at pH 10.0. The positive effect of calcium ion may be attributed to that the activity of cellulose synthase can be stimulated because the calcium inhibits an enzyme, which degrades the activator of the cellulose synthase in the membrane of *A. xylinum* (Aloni, Cohen, Benziman, & Delmer, 1983; Ross et al., 1985). The effect of calcium on the BC production in 8-day cultivations was also examined by using the NaOH-treated hydrolyzate, but no promising result was obtained. The reason why BC production using the $Ca(OH)_2$-treated hydrolyzate was improved should be related to both detoxification efficiency and other factors including supplement of calcium cation. NaOH treatment may not give a satisfactory detoxification effect.

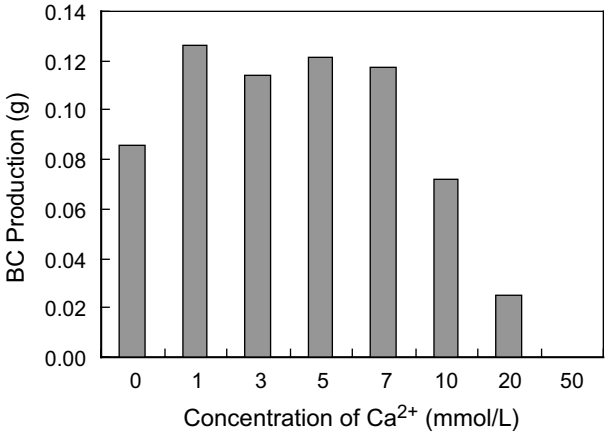

Fig. 1. Effect of Ca^{2+} concentration on BC production.

In various bacteria, the effect of Ca^{2+} is different. Calcium ion can affect not only the activity and stability of enzymes in some bacteria (Welfle, Misselwitz, Welfle, Politz, & Borriss, 1995) but also the cell surface electrostatics of some oral bacteria (Yamashita, Kunimori, & Takehara, 1991). Study on the complicated mechanisms behind the findings are still being on the way in our lab to find if possible stimulatory factors for the fermenting bacterium formed during the alkaline processing. In Jönsson's lab in Sweden, an investigation on possible stimulatory factors in alkaline-treated spruce hydrolyzates for fuel ethanol production is of great interest but the mechanism is still unclear (Persson et al., 2002).

4. Conclusions

The research applied different methods to detoxify hydrolyzates for BC production and the results indicated that detoxification with $Ca(OH)_2$ treatment worked better than that with NaOH. If adding activated charcoal or laccase in the processing, the detoxification effects would go further and BC production could be improved more. Based on same concentration of carbon sources, BC yield using these hydrolyzates as carbon sources was much higher than that using glucose or mannose. Moreover, the result also showed that detoxification treatments at pH 10.0 resulted in better fermentability than treatments at pH 5.0. The addition of extra calcium in glucose medium in the form of $CaCl_2$ at pH 5 did result in an improvement of less than 50% in BC production, which was not comparable to the $Ca(OH)_2$ treatments at pH 10.0.

It is the first time to develop the hydrolyzate of konjac powder as an excellent carbon source for BC production successfully in the world. The yield of BC production with the new carbon source was much better than those with conventional carbon sources, and the economic cost of the carbon source is relatively low because konjac is an economical and abundant resource in China and many other species of konjac have not been applied yet. The results

indicate that konjac powder could serve as a feedstock for bacterial cellulose production and cultivation of *A. rivieri Durieu* would bring more economic benefits to farmers in future.

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