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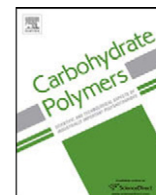

Statistical optimization of culture conditions for bacterial cellulose production by *Acetobacter xylinum* BPR 2001 from maple syrup

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ABSTRACT

Bacterial cellulose production by *Acetobacter xylinum* BPR 2001 was optimized using maple syrup as a carbon source. Twelve culture parameters were screened by the Plackett–Burman design and significant parameters were optimized by the response surface methodology using a three-level, four-factor Box–Behnken design. For fermentation in a rotary shaker, the optimal conditions for bacterial cellulose production were: maple syrup 30 g carbohydrate/l, $(NH_4)_2SO_4$ 3.3 g/l, KH_2PO_4 1 g/l, yeast extract 20 g/l, citric acid 1.6 g/l, trisodium citrate dehydrate 2.4 g/l, ethanol 0.5% (v/v), acetic acid 0.5 g/l, $MgSO_4{\cdot}7H_2O$ 0.8 g/l, inoculum age 3 days, inoculum volume 6% (v/v), shaking speed 135 rpm, and incubation temperature 25 °C. Comparison of bacterial cellulose production with maple syrup or pure sugars showed maple syrup was a suitable carbon source. This was the first demonstration of conversion of maple syrup, a plentiful renewable resource, into bacterial cellulose, a nanobiomaterial ideal for many applications.

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1. Introduction

Bacterial cellulose (BC), a polymer of glucose units linked together by β-(1-4)-glycosidic linkages, is produced as ~50 nm diameter nanofibers by the bacterium *Acetobacter xylinum* (Klemm et al., 2006). BC is distinguished from that of wood cellulose by its high purity, free from lignin and hemicellulose and its very high water content (Klemm et al., 2006). Bacterial cellulose has a high degree of crystallinity and mechanical properties superior to other celluloses (Guhados, Wan, & Hutter, 2005). This, coupled with the nanometer scale morphology, makes it ideal for a broad range of biomedical and other applications. Biomedical applications investigated include wound dressing (Czaja, Krystynowicz, Bielecki, & Brown, 2006), micro blood vessel (Klemm, Schumann, Udhardt, & Marsch, 2001), three-dimensional scaffold material for tissue engineering of bone and cartilage (Jiang et al., 2007; Svensson, Nicklasson, Harrah, Panilaitis, & Kaplan, 2005), and tissue repair (Christner, Wyrwa, Marsch, Kullertz, & Thiericke, 1999; Mello, Feltrin, Fontes Neto, & Ferraz, 1997; Suehiro et al., 2007). BC's hydrophilic nature and native dimensions make it an ideal material for the preparation of hydrophilic, biocompatible nanocomposites with controlled mechanical properties for cardiovascular devices (Million, Guhados, & Wan, 2008). Other applications investigated include acoustic diaphragm (Vandamme, De Baets, Vanbaelen, Joris, & De Wulf, 1998), membrane electrode assemblies in fuel cell (Evans, O'Neil, Malyvanh, Lee, & Woodward, 2003), optically transparent composites (Ifuku et al., 2007), and electronic paper displays (Shah & Brown, 2005). The success of these exciting applications depends on the ability to produce BC economically. Cultivation using novel bioreactors (Hornung, Ludwig, & Schmauder, 2007; Ishida, Sugano, Nakai, & Shoda, 2002; Kim, Kim, Wee, Park, & Ryu, 2007; Moon, Park, Chun, & Kim, 2006), and genetic manipulation (Chien, Chen, Yang, & Lee, 2006; Park, Kim, & Kim, 2006) have been reported for enhancement of BC productivity.

Traditional sources of carbon for microbial fermentation are sugars such as glucose, fructose and sucrose (Bae & Shoda, 2005a, 2005b). High production of BC has been achieved using fructose as a carbon source (Joseph, Rowe, Margaritis, & Wan, 2003). More recently, unconventional feedstocks from renewable resources and waste streams have been investigated. These include unamended food process effluents (Thompson & Hamilton, 2000), beet and sugar cane molasses (Bae & Shoda, 2005a, 2005b; Keshk & Sameshima, 2006), enzymatic treated food waste liquids (Park et al., 2006), acid hydrolyzed konjac powder (Hong & Qiu, 2008), saccharified solutions derived from sweet potato pulp (Shigematsu et al., 2005), hydrolyzed hemicelluloses from waste liquor of atmospheric acetic acid pulping (Uraki, Morito, Kishimoto, & Sano, 2002) and fruit juices (Kurosumi, Sasaki, Yamashita, & Nakamura, 2009). Of particular interest is maple sap, a renewable carbon source from

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the maple tree. Maple sap has traditionally been used for the production of syrup and recently 84% of the world's production of maple syrup is in Canada (Kurosumi et al., 2009). Maple syrup has a carbohydrate content of about 67% (w/w), of which 89% is sucrose and most of the remainder is fructose and glucose, making it an attractive carbon source for fermentations (Morselli, 1975). However, except for a recent investigation for the production of poly-β-hydroxybutyrate by *Alcaligenes latus*, little work has been done on its use as a renewable carbon source for microbial fermentation (Kurosumi et al., 2009).

The productivity of BC depends on culture conditions, which include cultivation method, composition of growth medium (Keshk & Sameshima, 2005), dissolved oxygen (Kouda, Naritomi, Yano, & Yoshinaga, 1997), temperature, pH of the growth medium (Noro, Sugano, & Shoda, 2004), inoculation ratio (Hutchens, León, O'neill, & Evans, 2007) and inoculum age (Park, Jung, & Park, 2003). The Plackett–Burman design and the response surface methodology (RSM) based on the Box–Behnken design can be used to optimize culture conditions (Plackett & Burman, 1946). RSM can be used for optimization of the culture conditions which are found to significantly affect BC production from the Plackett–Burman design, with a minimum number of experiments. With these experimental design approaches most of the disadvantages of conventional one-factor-at-a-time experiments can be avoided (Bae & Shoda, 2005a, 2005b; Box & Behnken, 1960; Naveena, Alaf, Bhadriah, & Reddy, 2005).

This study investigates the production of BC using maple syrup as the renewable carbon source compared to the traditional carbon sources of glucose, fructose and sucrose. Culture conditions affecting biomass and BC production are determined using Plackett–Burman design and optimized by the RSM.

2. Materials and methods

2.1. Microorganism and materials

A. xylinum BPR 2001 (ATCC #700178) was used in this study because it shows high BC production in agitated cultures (Toyosaki et al., 1995). Pure maple syrup (Kirkland Signature; No. 1 Light 100% pure maple syrup; batch 03082; lic. no. 3585; from Costco Wholesale Corporation, Ottawa, Ontario, Canada) was used as the carbon source in the culture medium and has a carbohydrate content of 54 g per 60 ml. Yeast extract powder (Y1001, HY-Yest®412), cellulase (C-0901, from *Penicillum funiculosum*), fructose, glucose, sucrose, ammonium sulfate, magnesium sulfate heptahydrate, and trisodium citrate dihydrate were purchased from Sigma–Aldrich, Ontario, Canada were of laboratory grade, and were used without further purification.

2.2. Culture conditions

The fructose based medium used for the inoculum had the following composition (per liter of deionized water): fructose 20 g, $(NH_4)_2SO_4$ 3.3 g, yeast extract 20 g, KH_2PO_4 1 g, $MgSO_4{\cdot}7H_2O$ 0.122 g. Without any buffer, the pH of the inoculum medium was 5.09 after autoclaving. To prepare the inoculum, *A. xylinum* BPR 2001 from an agar plate was transferred aseptically into a 500 ml Erlenmeyer flask containing 250 ml of inoculum medium and incubated at 27 °C in a shaker incubator (Model G-25R, New Brunswick Scientific Co., USA) set at 160 rpm. BC was produced in 500 ml Erlenmeyer flasks containing 250 ml of growth medium containing 3.3 g/l of $(NH_4)_2SO_4$, 1 g/l of KH_2PO_4 and maple syrup as carbon source under different conditions according to the experimental design (Tables 1, 2, 4 and 5).

2.3. Bacterial cellulose and cell dry weight determination

The fermentation broth was processed according to Joseph et al. (2003). For each determination two 5 ml samples of broth from shake flasks, containing between 1 and 20 mg of cellulose each, were homogenized at 12,000 rpm for 15 s with a laboratory blender (Model 51BL30, Waring Commercial, USA). To remove the culture broth, samples were centrifuged at 3000 rpm for 20 min, washed with distilled water and centrifuged again. The washing procedure was repeated three times for every sample. For bacterial cellulose yield determination, one washed sample was treated with 15 ml of 1% (w/v) sodium hydroxide at 90 °C for 30 min to dissolve the cells. The bacterial cellulose obtained was filtered, washed with distilled water, dried at 60 °C for 24 h, and weighed. For cell dry weight determination, the second washed sample was suspended in 10 ml of cellulase solution. The cellulase solution contained 2.5% (w/v) suspension of crude cellulase in 1 M citrate buffer (pH 5), and was filtered through a cellulose nitrate filter paper (0.45 μm pore size, Whatman) to remove suspended solids before combining with the sample. The mixture was kept at 37 °C for 2 h to hydrolyze the cellulose (Joseph et al., 2003). The sample was then filtered with a dried and weighed filter paper (same type as above) and washed. The filter paper with the biomass cake was dried at 60 °C for 24 h and weighed.

2.4. Screening of nutrients and physical parameters using the Plackett–Burman design

The Plackett–Burman design was used to identify factors that significantly influence BC production. A 24-run Plackett–Burman design (Plackett & Burman, 1946) was used to screen 12 variables including nutrients, buffer and physical parameters at high (+1) and low (−1) levels (Tables 1 and 2). Maple syrup and yeast extract were tested as carbon and nitrogen source for their impact on BC production. Their concentrations were set based on our experience using fructose as carbon source. The concentrations of ethanol, acetic acid and agar were set according to reports (Box & Behnken, 1960; Million et al., 2008; Toyosaki et al., 1995), at concentrations found to increase BC production. The concentration of $MgSO_4{\cdot}7H_2O$ was set at 0.8 g/l because BC production was increased at this magnesium concentration (Son et al., 2003). Decrease of pH by gluconic acid production can inhibit cellulose production (Hutchens et al., 2007). Therefore citric acid and tri-sodium citrate dehydrate were used as a buffer to maintain pH in the culture. The physical parameters of inoculum size and age, incubation period, shaking speed and temperature were also studied as possible factors influencing BC production.

In the 24-run Plackett–Burman design each row represents an experiment and each column represents an independent variable (Table 2). The signs +1 and −1 represent the high and low levels of the independent variables under investigation.

2.5. Optimal culture conditions by the Box–Behnken design

To determine the optimum levels of the most significant variables identified by the Plackett–Burman design the Box–Behnken method was used for experimental design and RSM was used to identify optimal conditions. Each significant variable was studied at three different levels (−1, 0, +1), where 0 represents the central value of each variable (Table 4). Table 5 shows the experimental conditions tested according to the Box–Behnken design. Less significant variables were set based on experimental data of the Plackett–Burman design or the basal medium composition. BC production was taken as response (Y) and culture conditions were taken as independent variables (X). The experimental results were fitted by regression to a predictive quadratic polynomial that gave

Table 1
Culture conditions and their levels in Plackett–Burman design for BC production in rotary shaker incubator.

Symbols designated	Variables	Lower level (−1)	Higher level (+1)
X_1	Maple syrup (g carbohydrate/l)[a]	20	40
X_2	Incubation period (day)	7	15
X_3	Yeast extract (g/l)	10	20
X_4	Citric acid and trisodium citrate dihydrate (g/l)	1.6 and 2.4	3.2 and 4.8
X_5	Ethanol (v/v, %)	0.5	1
X_6	Acetic acid (g/l)	0.5	1
X_7	$MgSO_4 \cdot 7H_2O$ (g/l)	0.4	0.8
X_8	Agar (g/l)	0	2
X_9	Size of inoculum (v/v, %)	3	6
X_{10}	Age of inoculum (day)	3	6
X_{11}	Temperature (°C)	25	30
X_{12}	Shaker speed (rpm)	140	170

[a] Calculated from the carbohydrate content of maple syrup (54 g/60 ml).

BC production (Y) as a function of culture conditions (X),

$$Y = A_0 + \sum_{i=1}^{n} A_i X_i + \sum_{i=1}^{n} A_{ii} X_i^2 + \sum_{i=1}^{n} \sum_{j=1}^{n} A_{ij} X_i X_j \quad (1)$$

where n is the number of investigated variables; X_i the value of the investigated variable; A_0 the offset term; A_i the ith linear coefficient; A_{ii} the ith quadratic coefficient; A_{ij} is the ijth interaction coefficient. Eq. (1) was used to predict the optimum values of the independent variables by setting the partial derivative with respect to each independent variable to zero.

2.6. Comparison of maple syrup, fructose, glucose and sucrose as carbon sources for bacterial cellulose production

Since the carbohydrates of maple syrup are predominantly sucrose, glucose and fructose (Uraki et al., 2002) BC production using these carbon sources was compared to BC production using maple syrup. Maple syrup concentration and other conditions were set at their optimum values. Sucrose, glucose and fructose concentrations were set at the same concentration in the medium as maple syrup carbohydrates (Fig. 4). A mixture of sucrose (89% of total sugar), fructose and glucose (5.5% of total sugar each) was tested as a simulated maple syrup for BC production.

2.7. Data analysis

Statistical experimental designs were generated and analyzed using ‘Minitab 15′ (Minitab Inc., USA) and SAS (SAS Institute Inc., USA). Three-dimensional surface plots were constructed for visualization of interaction between significant variables and their optimal values. All experiments were carried out independently in triplicates and the average values are presented.

3. Results

3.1. Screening of key culture parameters using the Plackett–Burman design

Results from the 24-run Plackett–Burman experimental design on the effects of the 12 culture parameters on BC and cell dry weight production are summarized in Table 2. Table 3 shows the regression analysis by ANOVA of the experimental responses to these 12 culture parameters in terms of BC and cell dry weight production. It can be seen that within the tested range maple syrup concentration, incubation period, size of inoculum and shaking speed had significant effect on BC production at a confidence level $P < 0.1$. For cell dry weight, the significant factors were incubation period, size of inoculum and shaking speed. In the determination of cell dry

Table 2
Plackett–Burman design for 12 variables with coded values and observed results for BC production and cell dry weight.[a]

Run	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	X_{12}	BC production (g/l)	Cell dry weight (g/l)
1	1	−1	−1	−1	−1	1	−1	1	−1	−1	1	1	0.41	1.72
2	1	1	−1	−1	−1	−1	1	−1	1	−1	−1	1	1.48	1.58
3	1	1	1	−1	−1	−1	−1	1	−1	1	−1	−1	0.77	1.96
4	1	1	1	1	−1	−1	−1	−1	1	−1	1	−1	1.49	1.32
5	1	1	1	1	1	−1	−1	−1	−1	1	−1	1	0.50	0.76
6	−1	1	1	1	1	1	−1	−1	−1	−1	1	−1	0.90	0.96
7	1	−1	1	1	1	1	1	−1	−1	−1	−1	1	0.64	0.44
8	−1	1	−1	1	1	1	1	1	−1	−1	−1	−1	0.50	1.96
9	1	−1	1	−1	1	1	1	1	1	−1	−1	−1	1.04	2.18
10	1	1	−1	1	−1	1	1	1	1	1	−1	−1	1.35	2.16
11	−1	1	1	−1	1	−1	1	1	1	1	1	−1	1.39	2.76
12	−1	−1	1	1	−1	1	−1	1	1	1	1	1	0.34	2.12
13	1	−1	−1	1	1	−1	1	−1	1	1	1	1	0.24	0.26
14	1	1	−1	−1	1	1	−1	1	−1	1	1	1	0.38	1.70
15	−1	1	1	−1	−1	1	1	−1	1	−1	1	1	0.59	1.04
16	−1	−1	1	1	−1	−1	1	1	−1	1	−1	1	0.36	0.88
17	1	−1	−1	1	1	−1	−1	1	1	−1	1	−1	0.50	1.70
18	−1	1	−1	−1	1	1	−1	−1	1	1	−1	1	0.24	0.86
19	1	−1	1	−1	−1	1	1	−1	−1	1	1	−1	0.51	0.88
20	−1	1	−1	1	−1	−1	1	1	−1	−1	1	1	0.37	0.58
21	−1	−1	1	−1	1	−1	−1	1	1	−1	−1	1	0.56	1.32
22	−1	−1	−1	1	−1	1	−1	−1	1	1	−1	−1	0.50	0.70
23	−1	−1	−1	−1	1	−1	1	−1	−1	1	1	−1	0.51	0.58
24	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	0.48	0.42

[a] Both are concentrations in culture broth.

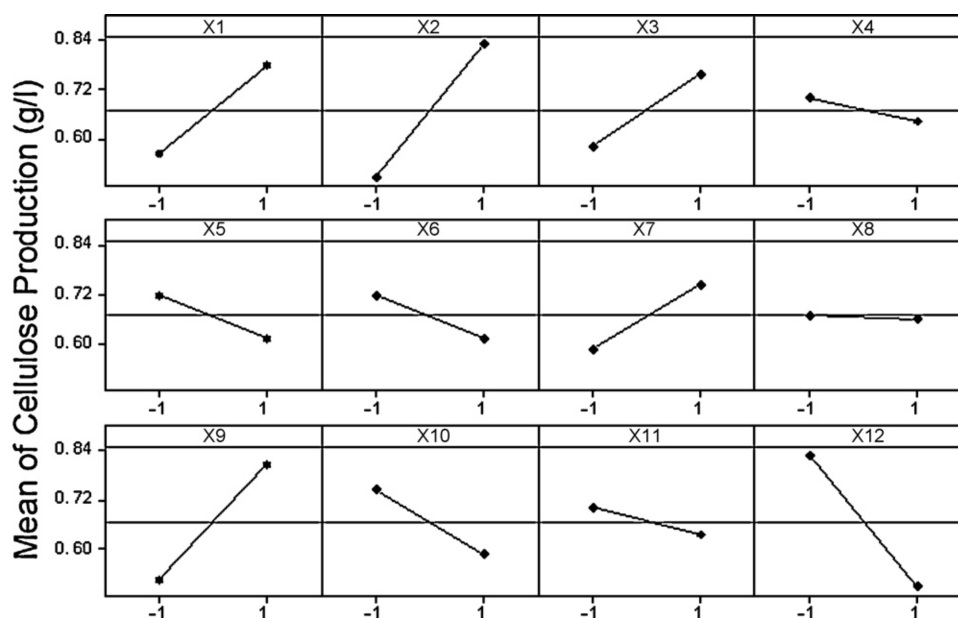

Fig. 1. Effects of 12 culture conditions on average BC production (X_1 to X_{12} are defined in Table 1).

Table 3
Results of regression analysis for the Plackett–Burman design.

Source	BC production (g/l)			Cell dry weight (g/l)		
	Effect	T	P	Effect	T	P
X_1	0.2142	1.84	0.093[a]	0.207	1.33	0.210
X_2	0.3225	2.77	0.018[a]	0.370	2.38	0.036[a]
X_3	0.1775	1.52	0.156	0.200	1.29	0.224
X_4	−0.0558	−0.48	0.641	−0.263	−1.70	0.118
X_5	−0.1042	−0.89	0.391	0.010	0.06	0.950
X_6	−0.1042	−0.89	0.391	0.217	1.40	0.190
X_7	0.1592	1.37	0.199	−0.020	−0.13	0.900
X_8	−0.0092	−0.08	0.939	0.937	6.03	0.000[a]
X_9	0.2825	2.42	0.034[a]	0.430	2.77	0.018[a]
X_{10}	−0.1558	−1.34	0.208	0.033	0.21	0.834
X_{11}	−0.0658	−0.56	0.583	0.033	0.21	0.834
X_{12}	−0.3192	−2.74	0.019[a]	−0.360	−2.320	0.041[a]

[a] $P < 0.1$ are considered significant.

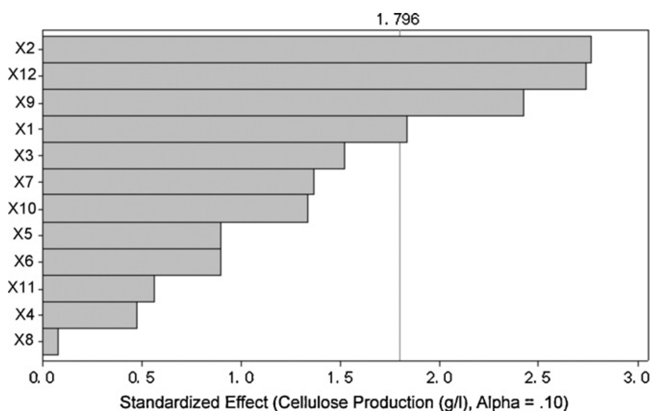

Fig. 2. Pareto chart of the standardized effects of 12 culture conditions on BC production (X_1 to X_{12} are defined in Table 1).

weight it was difficult to dissolve agar out of the cell cake with hot water. Although the data showed a significant effect of agar content on cell dry weight, it was not considered further since this may have been caused by inaccuracy in cell dry weight from residual agar. It is interesting to note that from the data high cell dry weight does not always result in high BC production, as shown in Table 2. By statistically analyzing the data in Table 2, two graphs (Figs. 1 and 2) were plotted using 'Minitab 15′ to visualize the results. Fig. 1 shows that the high level should be used for maple syrup, yeast extract, $MgSO_4{\cdot}7H_2O$, incubation period, and size of inoculum, and other factors fixed at the low level to increase BC production. Based on Fig. 2 maple syrup concentration, incubation period, size of inoculum and shaker speed were chosen as significant variables to be optimized. Other factors for optimization experiments were set as follows: $(NH_4)_2SO_4$ 3.3 g/l, KH_2PO_4 1 g/l, yeast extract 20 g/l, citric acid 1.6 g/l, trisodium citrate dehydrate 2.4 g/l, ethanol 0.5% (v/v), acetic acid 0.5 g/l, $MgSO_4{\cdot}7H_2O$ 0.8 g/l, age of inoculum 3 days and incubated at 25 °C.

3.2. Optimization of significant culture parameters with RSM based on the Box–Behnken design

The culture variables found to be significantly affect BC production were tested at values given in Table 4 for the Box–Behnken design, with other variables set to increase BC production based on the results presented in Fig. 1. The experimentally determined BC production, shown in Table 5, obtained from Box–Behnken design was fitted to a second-order polynomial equation,

$$\begin{aligned} Y = {} & 0.533713 - 0.07567X_1 + 0.00985X_2 - 0.02883X_3 \\ & + 0.039359X_4 + 0.000437X_1^2 + 4.17 \times 10^{-6}X_2^2 \\ & + 0.001991X_3^2 - 0.00017X_4^2 - 0.00012X_1X_2 - 6.7 \times 10^{-5}X_1X_3 \\ & + 6.33 \times 10^{-5}X_1X_4 + 0.000583X_2X_3 + 6.67 \times 10^{-5}X_2X_4 \end{aligned} \quad (2)$$

Table 4
Coded and actual values of the culture conditions tested in Box–Behnken design.

Factors	Symbols	Actual levels of coded factors		
		−1	0	+1
Maple syrup (g carbohydrate/l)[a]	X_1	30	55	80
Incubation period (day)	X_2	10	20	30
Size of inoculum (%, v/v)	X_3	3	6	9
Rotate speed (rpm)	X_4	110	140	170

[a] Calculated from the carbohydrate content of maple syrup (54 g/60 ml).

Table 5
Box–Bohenken design matrix for the four variables and experimental results.

Run	Carbohydrate of maple syrup (X_1)	Incubation period (X_2)	Size of inoculum (X_3)	Rotation speed (X_4)	BC production (g/l)
1	−1	−1	0	0	1.19
2	1	−1	0	0	0.23
3	−1	1	0	0	1.56
4	1	1	0	0	0.48
5	0	0	−1	−1	0.51
6	0	0	1	−1	0.5
7	0	0	−1	1	0.42
8	0	0	1	1	0.41
9	−1	0	−1	0	1.50
10	1	0	−1	0	0.34
11	−1	0	1	0	1.53
12	1	0	1	0	0.35
13	0	−1	0	−1	0.48
14	0	1	0	−1	0.64
15	0	−1	0	1	0.30
16	0	1	0	1	0.54
17	−1	0	0	−1	1.46
18	1	0	0	−1	0.32
19	−1	0	0	1	1.17
20	1	0	0	1	0.22
21	0	−1	−1	0	0.45
22	0	1	−1	0	0.88
23	0	−1	1	0	0.46
24	0	1	1	0	0.96
25	0	0	0	0	0.69
26	0	0	0	0	0.65
27	0	0	0	0	0.56

where Y represents BC production (g/l), and X_1, X_2, X_3 and X_4 are maple syrup concentration (g carbohydrate/l), incubation period (day), size of inoculum (%, v/v) and rotate speed (rpm), respectively.

The coefficients of the regression equation were calculated using the software SAS. The significance level of each variable was determined by t test, which showed that among the model terms X_1 (content of maple syrup), X_4 (shaking speed) and their quadratic term were significant with a probability of 99%, while the significance levels of other model terms were comparatively very low (data not shown). Statistical analysis using Fisher's test for ANOVA for the whole quadratic model of response surface is presented in Table 6. The regression model fit the data well, as shown by the significance level of 'total regress' ('Prob > F' = 0.0000), non-significance of 'lack of fit' ('Prob > F' = 0.4094) and the high value of R^2 (0.9812). Therefore the response equation is a suitable model for the optimization experiments within the tested ranges.

The optimum level of each variable and the effect of their interactions on BC production was visualized by plotting three dimensional response surface curves against any two independent variables, while keeping other variables at their middle levels (Fig. 3a and b). The lowest concentrations of maple syrup (30 g carbohydrate/l) and highest incubation times (30 days) increase BC production. The optimum shaker speed and inoculum size were predicted by the RSM to be close to the middle values, at 135 rpm and 6% (v/v), respectively (Fig. 3b). As Fig. 3a shows the effect of incubation period on BC production is small for periods between 10 and 30 days, and it was found to be insignificant among the model terms statistically analyzed by t test (data not shown). This variable had the most significant effect in screening experiments when periods as short as 7 days were used and there-

Table 6
Analysis of variance (ANOVA) for response surface quadratic model obtained from experiments to maximize BC production.

Source	DF	Degrees of squares	F-ratio	Prob > F
Regression				
Linear	4	3.866500	130.8	0.0000
Quadratic	4	0.750680	25.389	0.000
Crossproduct	6	0.015550	0.351	0.8962
Total regress	14	4.632730	44.768	0.0000
Residual				
Lack of fit	10	0.079833	1.801	0.4094
Pure error	2	0.008867		
Total error	12	0.088700		
R^2	0.9812			

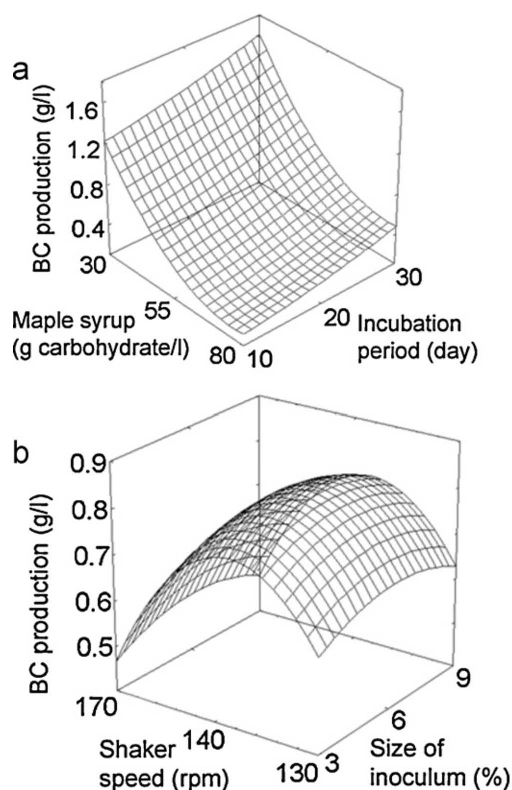

Fig. 3. (a) Response surface curve for BC production as a function of maple syrup concentration and incubation period. (b) Response surface curve for BC production as a function of shaker speed and size of inoculum.

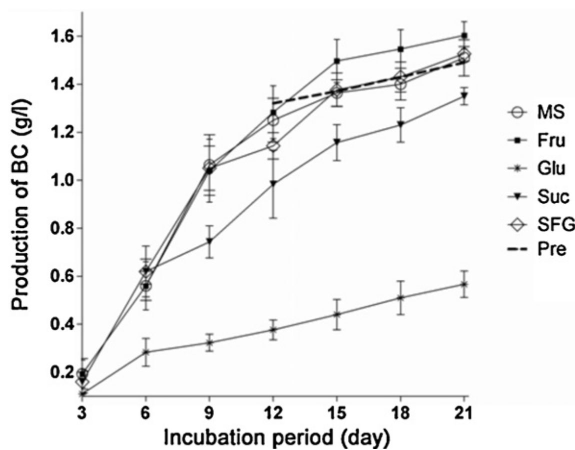

Fig. 4. Comparison of maple syrup with other carbohydrates for BC production, all at 30 g/l (MS, maple syrup; Fru, fructose; Glu, glucose; Suc, sucrose; SFG, mixture of sucrose (89%), fructose (5.5%) and glucose (5.5%); Pre, predicted response. Experimental values are means ± SD.

fore an incubation period of 10 days should be sufficient. Thus, the optimum conditions for BC production are: maple syrup 30 g carbohydrate/l, inoculum size 6% (v/v), shaker speed 135 rpm, and other factors as determined from the Plackett–Burman screening experiments.

3.3. Comparison of maple syrup with other carbohydrates and verification of the optimal conditions for BC production

The validity of experimental model and regression equation was tested by producing BC at the calculated optimal conditions. Sucrose, glucose, fructose and their mixture, were used instead of maple syrup to show that maple syrup was a suitable carbon source. BC production with these carbon sources and predicted production from the second order regression equation are shown in Fig. 4. Since BC production was so slow after 15 days cultivation was stopped at day 21. The predicted production of BC was very close to the actual production between 12 and 21 days, which helps to validate the model. Compared to other carbon sources BC production using glucose was very low. The simulated maple syrup sugar mixture gave very similar production to maple syrup, and BC production was only slightly higher when fructose was used. Sucrose showed lower BC production than maple syrup but still much higher production than glucose.

4. Discussion

Many kinds of carbon sources derived from agricultural wastes and byproducts have been tested for production of BC. However, most of these raw materials must be hydrolyzed with acid (Bae & Shoda, 2005a, 2005b; Hong & Qiu, 2008; Park et al., 2006; Uraki et al., 2002) or enzymatically (Park et al., 2006; Shigematsu et al., 2005) before their use in fermentation. In the present study, untreated maple syrup was successfully used for BC production. BC production with maple syrup was almost as high as when using pure fructose, and much higher than when using glucose (Fig. 2). This is consistent with Bae's viewpoint that the composition of sugars do not affect BC production if fructose exists in the medium (Bae & Shoda, 2005a, 2005b), possibly because fructose activates a phosphoenolpyruvate-dependent phosphotransferase system in *A. xylinum* (Tonouchi, Tsuchida, Yoshinaga, Beppu, & Horinouchi, 1996). From the response surface curve for BC production it is seen that 30 g/l of carbohydrate resulted in higher BC production than for higher carbohydrate concentrations (Fig. 1a). In batch fermentation with molasses as carbon source for BC production 20 g/l carbohydrate was found to give the maximum BC production compared to higher carbohydrate concentrations (Bae & Shoda, 2004). The results presented here support the finding that higher sugar concentrations decrease BC production. Using maple syrup with a lower carbohydrate concentrations might further increase BC production.

Medium pH control was found to be critical for high BC productivity by *A. xylinum* in this and many previous studies (Joseph et al., 2003; Park et al., 2006). Oxidization of glucose by membrane-bound glucose dehydrogenase forms gluconic acid causing pH to decrease to as low as 3, which may inhibit BC production (Krystynowicz et al., 2002). In this study when glucose was used as a carbon source in buffered media the pH decreased to 3.7. Others have solved this problem by using corn steep liquor in the medium, which has some buffering capacity (Noro et al., 2004). When using carbon sources with sucrose the pH of buffered media only decreased to 4.1–4.9 during incubation (data not shown), while the optimum pH range for *A. xylinum* BPR2001 is 4.5–5.5 (Noro et al., 2004).

In order to overcome the mass transfer limitations in static culture BC was produced in a rotary incubator. These conditions favor non-cellulose producing mutants of *A. xylinum* which can quickly take over the culture, severely reducing BC production (Krystynowicz et al., 2005). In this study, the optimum shaking speed was found to be 135 rpm, and BC production declined significantly at 170 rpm. Contrary to expectations inoculum size did not significantly influence BC production within the tested range. Hutchens et al. (2007) hypothesized that the bacteria concentration would reach a constant equilibrium level irrespective of the initial inoculation concentration.

It was believed that addition of ethanol to the culture medium would improve BC production by functioning as an energy source instead of glucose (Naritomi, Kouda, Yano, & Yoshinaga, 1998; Yunoki, Osada, Kono, & Takai, 2004), and causing aggregation of BC which could reduce shear stress (Park, Park, & Jung, 2003). Acetic acid added in medium would be catabolized causing an increase in pH of the media, which may counteract the pH decrease due to gluconic acid formation (Vandamme et al., 1998). In the present study ethanol and acetic acid did not show significant effects within the tested concentration range when screened by the Plackett–Burman experiment. It is likely that effect of ethanol on shear and hence on the advantage for non BC producing mutants was much small compared to the effect of shaking speed. The effect of acetic acid on pH was probably overshadowed by the buffer used in our experiments.

Addition of water-soluble polymers such as agar (Bae, Sugano, & Shoda, 2004), sodium alginate (Zhou, Sun, Hu, Li, & Yang, 2007), xanthan (Chao, Mitarai, Sugano, & Shoda, 2001), polyacrylamide-co-acylic acid (Joseph et al., 2003), and acetan (Ishida et al., 2002) was reported to reduce shear in the medium reducing the selective advantage for non-BC producing mutants and also to reduce pellicle size and therefore increase mass transfer. Despite agar addition BC tended to form large irregular pellicles. In the lightly shaken Erlenmeyer flasks shear is probably much lower than in turbine stirred fermenters, where others found polymer addition to be important for BC production (Joseph et al., 2003). Others found magnesium increased BC production (Son et al., 2003) and in this study the high level of 0.8 g/L $MgSO_4{\cdot}7H_2O$ was found to be most suitable for BC production.

BC production requires good oxygen transfer but is not increased by higher oxygen pressures (Hornung, Ludwig, Gerrard, & Schmauder, 2006; Kouda et al., 1997). With bubbled air supply in turbine stirred fermenters or airlift bioreactor, BC production of *A. xylinum* BPR 2001 can reach 6–8 g/l in 3 days of fermentation (Bae & Shoda, 2005a, 2005b; Ishida et al., 2002).

5. Conclusions

In the present study, we have demonstrated that, using the response surface methodology approach for optimization, BC can be produced in shake flask culture at a rate of 1.51 g/l with maple syrup as carbon source, which is comparable to that using pure fructose as the carbon source (1.60 g/l). Therefore maple syrup can be used effectively as an alternative to fructose in commercial scale BC production. In major maple syrup producing countries such Canada and the United States, the use of maple sap collected from the trees directly for fermentation is even more attractive. Canada alone produces more than 80% of the world's maple syrup, about 7 million gallons in 2005 and there is room for further expansion of its production (Keough, 2009). This represents an effective use of a plentiful and renewable resource in the form of either maple syrup or maple sap for the production of a nanobiomaterial suitable for a broad range of biomedical and other applications.

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