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Effect of media components on cell growth and bacterial cellulose production from *Acetobacter aceti* MTCC 2623

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ABSTRACT

Acetobacter aceti MTCC 2623 was studied as an alternative microbial source for bacterial cellulose (BC) production. Effect of media components on cell growth rate, BC production and cellulose characteristics were studied. FTIR results showed significant variations in cellulose characteristics produced by *A. aceti* in different media. Results have shown the role of fermentation time on crystallinity ratio of BC in different media. Further, effect of six different media components on cell growth and BC production was studied using fractional factorial design. Citric acid was found to be the most significant media component for cell growth rate (95% confidence level, $R^2 = 0.95$). However, direct role of these parameters on cellulose production was not established (p-value > 0.05).

1. Introduction

Cellulose, a homopolymer of β (1–4) linked glucose, is the most abundant macromolecule on earth with vascular plants being the major source (Brown, 2004). Cellulose from higher vascular plants has been used in the manufacturing of paper and textiles for millenia (Hon, 1994). However, the growing industrial demand for cellulose based products has imposed extreme negative pressure on the delicate ecological balance of the plant world causing deforestation and creating global environmental issues (Park, Park, & Jung, 2003). A great deal of interest has been created worldwide on the production of cellulose by using alternative sources, to reduce the environmental impact to a minimum. Existing reports suggest that bacterial cellulose (BC) may be a better choice for manufacturing cellulose based products (Castro et al., 2011). BC is one of the most promising biological materials, which displays many unique properties over plant cellulose *viz.* higher purity, crystallinity, water absorption, tensile strength, low degree of polymerization and stronger biological adaptability compared to natural plant cellulose (Bäckdahl et al., 2006; Klemm et al., 2006). Such biopolymeric material have applications in different commercially important sectors such as food, textile, paper, composite membranes, medicine, biomedical material, bioadsorbent material and loud speaker diaphragms (Oshima, Taguchi, Ohe, & Baba, 2011; Svensson et al., 2005; Wan et al., 2006). Studies have also reported the possible application of BC as a scaffold material (Bäckdahl, Esguerra, Delbro, Risberg, & Gatenholm, 2008). Bacterial strains reported for BC production under optimum bioprocess conditions are *Sarcina*, *Agrobacterium*, *Rhizobium*, *Acetobacter*, *Achromobacter*, *Aerobacter*, *Azotobacter*, *Salmonella* and *Escherichia* (Moosavi-Nasab & Yousefi, 2011; Naritomi, Kouda, Yano, & Yoshinaga, 1998; Sani & Dahman, 2010). *Acetobacter xylinum* is one of the most extensively studied sources of BC (Naritomi et al., 1998; Sheykhnazari, Tabarsa, Ashori, Shakeri, & Golalipour, 2011; Zeng, Small, & Wan, 2011). The extensive research on enhanced production of BC from *A. xylinum*, using bioprocess condition optimization and detailed interpretation of cellulose biosynthesis pathways have provided a wealth of information on BC, but limited reports are available on BC production from alternative *Acetobacter* strains, that may also be of commercial importance. Therefore, the goal of this study was to study the effect of media and culture conditions on BC production from alternative cellulose producing strain *Acetobacter aceti* MTCC 2623. This work was undertaken with following objectives: (1) to screen different media for cell growth and cellulose production, (2) characterization of BC using FTIR and (3) fractional factorial design approach to study the significance of media components on cell growth and cellulose production. In order to compare the

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production of BC from *A. aceti* MTCC 2623 with *A. xylinum*, initial fermentation studies were performed in media documented for *A. xylinum*. Moreover, no studies focused on the characteristics of BC from *A. aceti* MTCC 2623 have been published.

To the best of our knowledge, this is the first report on the effect of different media components on growth of *A. aceti* MTCC 2623, BC production and its structural arrangement thereof.

2. Materials and methods

2.1. Bacterial strain and culture conditions

A. aceti MTCC 2623 used in this study was procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. The cell suspension was stored at −80 °C in a 20% glycerol solution. To revive the culture, 100 μl of cell suspension stored at −80 °C was added to 50 ml of revival medium and cultivated at 30 °C and 140 rpm for 2 days. The revival medium consisted of following constituents per liter: 5.0 g of yeast extract, 3.0 g of peptone and 25 g of mannitol. Culture grown in this media was used as inoculum for further studies.

2.2. Medium composition and cultivation

All the chemicals used were of analytical grade and commercially available, unless specified. Cell growth and BC production were studied in three different media. The three different media used were designated M1 (2% of glucose, 0.5% of yeast extract, 0.5% of peptone, 0.27% of disodium phosphate and 0.15% of citric acid) (Setyawati, Chien, & Lee, 2007), M2 (2.5% of mannitol, 0.3% of tryptone, 0.5% of yeast extract) (Wei, Yang, & Hong, 2011) and M3 (2.5% of glucose, 0.75% of yeast extract, 1% of peptone, 1% of disodium phosphate and 1% of acetic acid) (Tang, Jia, Jia, & Yang, 2010). Fermentation studies were performed in 150 ml culture media inoculated with 1% (v/v) cell suspension for 24 h at 30 °C and 140 rpm.

2.3. Analytical methods

10 ml of culture broth was recovered at regular time intervals, to determine cell growth and BC concentration. Cell concentration was estimated by measuring the optical density at 600 nm (OD_{600}) after cellulase treatment of culture broth and bacterial growth was estimated as specific growth rate (h^{-1}) (Zeng et al., 2011). BC was purified and quantified from the harvested broth samples by alkali treatment (Cheng, Catchmark, & Demirci, 2009). All experiments were performed in triplicate with mean value of the observations being used for calculations.

2.4. FTIR analysis

BC was characterized using FTIR spectroscopy. Sample pellets were prepared by mixing equal amount of cellulose samples with spectroscopic grade KBr (1:100) and subsequently pelletizing the mixture at 10 Torr. The vibrational modes existing in BC samples were probed by Perkin-Elmer *BXII FTIR* spectrophotometer (Perkin-Elmer, USA). The absorbance mode FTIR data were recorded with 2 cm^{-1} resolution in the wave number region of 4500–600 cm^{-1}. Commercially available microcrystalline cellulose (CDH, India) was used as standard.

2.5. Screening of media components using fractional factorial design

Fractional factorial design was used to study the significance of different media components on BC production. A 8-run fractional factorial experiment, designed using Minitab® 15 and SAS® were used to screen six variables at high (+1) and low (−1) levels (Table 1). The choice of media components was based on previous reports on BC production from *Acetobacter* sp. (Setyawati et al., 2007; Tang et al., 2010; Wei et al., 2011). In the 8-run fractional factorial design, each row represents an experiment and each column represents an independent variable (Table 2). Cell growth rate and BC concentration were taken as responses.

Table 1
Nutrient components and their levels in fractional factorial design for BC production.

Symbol designated	Variables	Lower level (−1)[a]	Higher Level (+1)[a]
X1	Mannitol	0	2.5
X2	Yeast extract	0	0.5
X3	Tryptone	0	0.3
X4	Galactose	0	2.0
X5	Disodium phosphate	0	0.27
X6	Citric acid	0	0.15

[a] All components are measured as % (w/v).

3. Results and discussion

Comparison of *A. aceti* MTCC 2623 fermentation in three different media showed variation in specific growth rate and cellulose production (Table 2). Decrease in cellulose production was associated with decreased specific growth rate of *A. aceti* MTCC 2623. Culture showed log phase of cell growth during first 10 h of fermentation followed by deceleration phase (data not shown). Moreover, cellulose production was higher after 24 h of fermentation than at 6 h. Effect of media components and bioprocess conditions on BC production have been reported with different *Acetobacter* sp. (Hong & Qiu, 2008; Panesar, Chavan, Chopra, & Kennedy, 2012; Zeng et al., 2011). However, limited reports are available on effect of bioprocess parameters on production and characterization of BC from *A. aceti* MTCC 2623 (Panesar et al., 2012). Improved BC production, compared to existing report showed the significance of media composition in affecting cellulose production. Results have also shown the significance of fermentation time and media composition on BC characteristics, analyzed using FTIR.

3.1. FTIR analysis

FTIR spectroscopic investigations evidenced the capability of different infrared active adsorption bands to characterize the ordering degree of cellulose polymer (Fig. 1a–c).

The two major allomorphs of this biopolymer, *viz.* cellulose I and cellulose II can be easily analyzed based on FTIR bands and crystallinity ratios. Cellulose I, the dominant form in nature, consists of a crystalline arrangement of linear β-1,4-glucan chains, all of which are oriented parallel to one another with the same polarity. The extended chain conformation of cellulose I is responsible for the extraordinary mechanical strength. Normally, cellulose II is formed from cellulose I through chemical treatments that alter the

Table 2
Specific growth rate and bacterial cellulose production by *A. aceti* MTCC 2623 during screening of media after 6 and 24 h fermentation.

Media	Amount of cellulose pellet (mg/ml)		Specific growth rate (h^{-1})
	After 6 h	After 24 h	
M1	2.05 ± 0.17	3.20 ± 0.15	0.1625
M2	0	3.9 ± 0.08	0.1979
M3	0	0	0.061

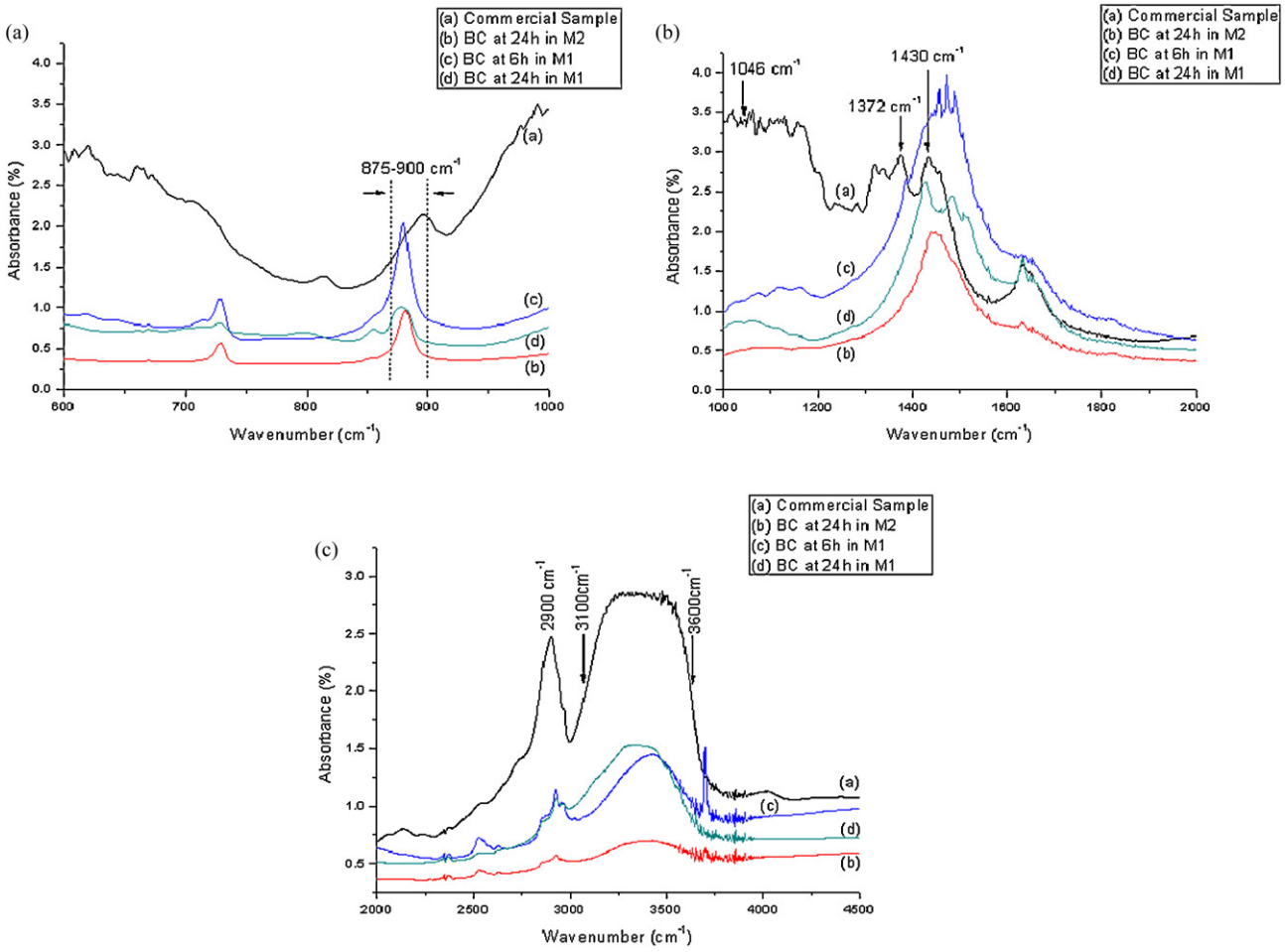

Fig. 1. FTIR spectra of commercial cellulose and BC produced in different media at different time intervals: (a) absorption spectra from 600 to 1000 cm^{-1}, (b) absorption spectra from 1000 to 2000 cm^{-1}, and (c) absorption spectra from 2000 to 4500 cm^{-1}.

crystal structure (*e.g.*, mercerization) (Lee, Brown, Kuga, Shoda, & Kobayashi, 1994).

Our FTIR results showed that the characteristic vibrational modes of BC produced were almost same in the typical fingerprint regions as reported earlier (Gea et al., 2011; Huang, Chen, Lin, Hsu, & Chen, 2010). Comparison of FTIR spectra in the range of 4500–600 cm^{-1} of BC with commercial cellulose revealed the existence of characteristic vibrational region of C—H out of plane bending (900–870 cm^{-1}) (Fig. 1a) (Gea et al., 2011). The FTIR absorption band at 892 cm^{-1}, assigned to C—O—C stretching at β (1–4) linkage, is designated as an "amorphous" absorption band. This band showed increase in the intensity in the BC samples (Goh et al., 2012). FTIR analysis indicated that BC at 6 h in media M1 is more amorphous compared to BC at 24 h in both media M1 and M2. This suggests the possible role of media components and fermentation time on changing cellulose structure from amorphous to crystalline domain. The absence of any other non-characteristics bands in BC samples compared to commercial cellulose samples also confirms the purity of BC and its possible application in food and non food products (Bertocchi, Delneri, Signore, Weng, & Bruschi, 1997). Existence of characteristic bands at 1046 cm^{-1} and 1071–1067 cm^{-1}, revealed the bending of C—O—H bond of carbohydrates (Fig. 1b). Absorption peak around 1111 cm^{-1}, another signature band of cellulose, corresponds to C—C bonds of the monomer units of polysaccharide. FTIR spectroscopy data revealed that a weak band in the range of 900–870 cm^{-1} and a strong band at 1430 cm^{-1} define the cellulose allomorph as cellulose I (Nelson & O'Connor, 1964). The absorbance band at 1430 cm^{-1} is assigned to symmetric CH_2 bending vibration. Moreover, the intensity of the infrared band at 1430 cm^{-1} has also been correlated with the degree of crystallinity and is also referred as "crystallinity band" (Bertocchi et al., 1997; Ciolacu, Ciolacu, & Popa, 2011). Measurement of crystallinity of cellulosic materials was performed by means of ratio of crystallinity (Cr.R) (Table 3). Ratio of crystallinity, documented in previous report, for different cellulosic samples, are the absorbance ratios from 1372 to 2900 cm^{-1} ($Cr.R_1$) and absorbance ratio from 1430 to 893 cm^{-1} ($Cr.R_2$) (Ciolacu et al., 2011). The signature band at 2900 cm^{-1} attributing to C—H stretching vibration confirm amorphous characteristic of cellulose. Band indicate a shift of 27 cm^{-1}, to the higher wave number values compared to commercial cellulose, along with a strong decrease in the intensity of this band. Furthermore, the relative magnitudes of absorption intensities indicate commercial cellulose to be more amorphous than BC. The broad band in the 3600–3100 cm^{-1} due to OH-stretching vibration,

Table 3
Ratios of crystallinity (Cr.R) of cellulose samples at different fingerprint regions of FTIR.

Sample	$Cr.R_1$ [a]	$Cr.R_2$ [b]
Commercial cellulose	1.40	0.73
BC in media M1 at 6 h	3.24	3.50
BC in media M1 at 24 h	4.03	4.21
BC in media M2 at 24 h	3.71	4.06

[a] Absorbance ratio (A_{1430}/A_{892}).
[b] Absorbance ratio (A_{1372}/A_{2900}).

Table 4
Fractional factorial design of experiment to study significance of media component on BC production by *A. aceti* MTCC 2623.

Experimental run	Mannitol (X1)	Yeast extract (X2)	Tryptone (X3)	Galactose (X4)	Disodium phosphate (X5)	Citric acid (X6)	Response (*Y*)	
							Specific growth rate (h^{-1})	Pellet (mg/ml)
1	−1	−1	−1	+1	+1	+1	0.62	No pellet
2	+1	−1	−1	−1	−1	+1	0.14	No pellet
3	−1	+1	−1	−1	+1	−1	0.06	No pellet
4	+1	+1	−1	+1	−1	−1	0.16	No pellet
5	−1	−1	+1	+1	−1	−1	0.07	No pellet
6	+1	−1	+1	−1	+1	−1	0.007	0.4
7	−1	+1	+1	−1	−1	+1	0.27	No pellet
8	+1	+1	+1	+1	+1	+1	0.10	3.5

Table 5
Regression analysis for fractional factorial design.

Component	Specific growth rate (h^{-1})			BC production (mg/ml)		
	Effect estimate	*t* ratio	*p* value[#]	Effect estimate	*t* ratio	*p* value[#]
X1	0.33	84.9	0.0075	1.95	1.25	0.4276
X2	−0.22	−56.6	0.0113	1.55	1.00	0.5000
X3	0.37	94.6	0.0067	1.95	1.25	0.4276
X4	−0.35	−88.3	0.0072	1.55	1.00	0.5000
X5	0.30	76.2	0.0083	1.95	1.25	0.4276
X6	−0.53	−134.05	0.0047	1.55	1.00	0.5000

[#] $p < 0.05$ are considered significant.

gives considerable information concerning the hydroxyl bonds present in these cellulosic samples. The intramolecular hydrogen bonding of O(2)H···O(6) and O(3)H···O(5), and the intermolecular hydrogen bonding of O(6)H···O(3) in cellulose are generally shown at 3455–3410, 3375–3340 and 3310–3230 cm^{-1}, respectively (Ciolacu et al., 2011).

Analysis of ratio of crystallinity revealed that $Cr.R_1$ of commercial cellulose is significantly lower than bacterial cellulose, proposing bacterial cellulose to be more crystalline than commercial counterpart. Higher absorbance at 2900 cm^{-1}, the designated "amorphous" absorption band proposed lower crystallinity of commercial cellulose compared to BC with concomitant higher absorbance band at 1372 cm^{-1} also. Lower absorbance at 2900 cm^{-1} from BC samples suggested lower C—H bond stretching leading to amorphous arrangement of the polymer. Similarly $Cr.R_2$ analysis revealed higher absorbance at 893 cm^{-1}, another designated amorphous absorption band in commercial cellulose than BC which decreases $Cr.R_2$. Results propose comparatively less crystallinity in the spatial arrangement of the chemical structure of commercial cellulose compared to BC. However, the role of NaOH in changing one allomorph of cellulose to another (mercerization) cannot be ruled out (Oh, Yoo, Shin, & Seo, 2005). These estimations showed that ratio of crystallinity vary with media composition and fermentation time, proposing their role on spatial arrangement of BC. On the basis of these results, it can also be concluded that cellulose produced by the bacterial strain is more crystalline than commercial cellulose.

3.2. Screening of media components using fractional factorial design

Results from 8 run fractional factorial design on specific growth rate of *A. aceti* and BC production, are summarized in Table 4. Results showed that responses *viz.* specific growth rate and BC concentration showed no linear mathematical correlation with each other. Table 5 showed the regression analysis of the experimental responses to media components in terms of growth rate and BC production from *A. aceti*. Statistical analysis also showed that within the tested range, all six media components had significant effect on growth of *A. aceti* at confidence level $p < 0.05$, with citric acid being the most significant component. This can be attributed to the assimilation of citric acid as carbon source by *A. aceti* (Gromet-Elhanan & Hestrin, 1963). Moreover, citric acid is also responsible for maintaining pH of media for BC production from *Acetobacter* sp. (Hwang, Yang, Hwang, Pyun, & Kim, 1999; Ishihara, Matsunaga, Hayashi, & Tisler, 2002). However, significance of media component on BC production cannot be well predicted in the tested range ($p > 0.05$). Thus, it can be proposed that BC production in the experiment is an outcome of complex cellular metabolism in a particular media and cannot be directly linked with cell growth.

Based on fractional factorial design experiment, a linear mathematical model has been proposed (Eq. (1)),

$$Y = 0.17 - 0.076X1 - 0.030X2 - 0.06X3 + 0.059X4 + 0.02X5 + 0.1X6 \quad (1)$$

where *Y* is the response (specific growth rate) and *X* denotes media components under study (Table 1). The proposed mathematical model was confirmed using a randomly designed experiment with following compositions *viz.* X1 (−1), X2 (+1), X3 (−1), X4 (+1), X5 (+1) and X6 (+1), where values in parentheses are the coded values of six different selected components. Specific growth rate of *A. aceti* in this media was experimentally determined to be $0.38 \pm 0.001\ h^{-1}$, while that predicted using mathematical model was 0.45 h^{-1}. Moreover, the yield of BC obtained after 24 h fermentation was 3.5 mg/ml. Previous studies have shown the successful application of statistical approach for optimization of media for improving BC production from *A. aceti* MTCC 2623 (Panesar et al., 2012).

4. Conclusion

Fermentation studies with *A. aceti* MTCC 2623 and rigorous FTIR analysis demonstrated the significant effect of media components on the structural and spatial arrangement of BC. Role of media components on spatial arrangement of BC proves the complexity of metabolic pathways for cellulose production. Availability of limited reports improves the significance of such studies in proposing

the role of fermentation condition on BC characteristics. Further, fractional factorial design showed the significance of six different components on the growth rate of *A. aceti* with citric acid being most significant for cell growth. We anticipate that this study will pave the way for further optimization for enhancing BC production from *A. aceti* MTCC 2623, and its possible application in food and biopharmaceutical sector.

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