Research article

Bacterial Cellulose Production by *Acetobacter aceti* MTCC 2623 Using Different Carbon Sources

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Abstract

Cellulose is a naturally occurring polysaccharide that is produced by both plants and micro-organisms. It is one of the most prevalent organic substances in the natural world. Compared to plant cellulose, bacterial cellulose (BC) exhibits superior physicochemical properties such as high purity, crystallinity, biocompatibility, biodegradability, and water holding ability, making it an excellent choice of material for various industrial and biomedical applications. However, large scale production and utilization of BC suffers from limitations related to its low yield and high production cost. In this research, an attempt was made to investigate the effects of different carbon sources on the bacterial cell growth kinetics, BC yield and physicochemical characteristics of *Acetobacter aceti* MTCC 2623. The results showed variations in growth kinetics and BC yields under different conditions. Among the selected carbon sources, Hestrin and Schramm (HS) medium supplemented with glucose gave highest BC yield of 2.89±0.18 g/L, whereas glycine resulted in the lowest BC yield, which was 0.089±0.17 g/L. FTIR spectral analysis showed no significant variations in the characteristic vibrational bands for BC prepared using different carbon sources. DSC results indicated the superior thermal stability of BC samples over commercial cellulose. Our findings suggest that BC production by *A. aceti* using glucose as C-source in HS media may be scaled up for enhanced production of BC. The improved physicochemical characteristics of BC compared to commercial cellulose point to the importance of BC as a potential candidate for several biomedical and industrial applications.

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1. Introduction

Cellulose is the most abundant natural polymer found on the earth. It can be obtained from a variety of sources including plant cell wall, several species of bacteria, algae and some tunicates [1]. Structurally, cellulose is composed of repeating glucose units connected via glycosidic linkages. Plant cellulose is ubiquitous and generally exists as a lignocellulose complex, the extraction of which involves harsh acid and alkali treatment [2]. Increased industrial demand for cellulose derivatives resulted in significant demand for plant biomass, leading to negative ecological impacts [3]. BC is an excellent alternative to plant cellulose as it is naturally free from impurities such as lignin and wax [4]. This inherent abundance of natural polymer paves the way for exploring diverse applications of this novel biomaterial. BC is an extracellular polysaccharide produced by several bacterial species including *Salmonella, Sarcina, Agrobacterium, Acetobacter, Rhizobium*, and *Azotobacter*. *Acetobacter xylinum* is a well-known strain for the production of crystalline cellulose [5]. BC is an ultra-fine nanofibrillar, biomimetic biomaterial with excellent physicochemical properties such as high crystallinity and water holding capacity, high degree of polymerization, high flexibility and superior mechanical characteristics [5-7]. Due to these exclusive features, BC attracts interest for various industrial and biomedical applications such as implants and scaffolds, drug delivery, tissue regeneration, and wound healing [3-5].

BC synthesis rates and structural properties may come from the varying molecular weights, chemical structures, and bioavailability of carbon sources [8-10]. Major challenges in the large-scale production of BC for diverse applications are low productivity and high production cost. Factors reported to affect BC production include selection of an appropriate microbial strain, carbon sources and process condition optimization. BC characteristics and BC production rates show significant variations across various bacteria [11, 12]. High cellulose yielding microorganisms as well as production strategies are significant parameters known to affect BC yield and its physicochemical characteristics [12]. Therefore, understanding the metabolic transformation pathways and utilization rates of various carbon sources in a particular bacterium that produces BC could provide good opportunities for BC production optimization [13, 14].

BC production is done through microbial fermentation under static and agitated culture conditions [6, 8, 14-17]. The choice of cultivation method is known to influence the physical and mechanical properties of BC. Nutrient availability and its assimilation by microbial cells are known to affect the BC production rate and its characteristics. For cost-effective production of BC, studies have been carried out on exploring the role of carbon source on BC yield [15, 18-20]. Hestrin and Schramm (HS) is the most commonly used medium for BC production [21, 22]. Type of culture medium used for BC production accounts for around 30% of the total cost of BC production and its composition impacts the production efficiency for a given microbial strain [13].

The objective of the study was to investigate the effect of different carbon sources on cellulose yield by *A. aceti* MTCC 2623 under submerged fermentation condition. Physicochemical characterization of the produced BC samples was done using FTIR (Fourier Transform Infrared Spectroscopy) and DSC (differential scanning calorimeter). To the best of our understanding, this study is the first report to examine the role of different carbon sources on BC production by *A. aceti* and the effects on its physicochemical characteristics.

2.   Materials and Methods

2.1 Procurement of bacterial strain and its revival

Strain *A. aceti* MTCC 2623 was procured from the Microbial Type Culture Collection (MTCC), IMTECH, Chandīgarh, India. The bacterial strain was inoculated in HS medium. Cell suspension was cryopreserved in 10% glycerol at -80°C for further use. The cell suspension (100 µL) was added to 50 mL of HS (Hestrin and Schramm) medium for culture revival and was used subsequently in our study.

2.2 Media composition and cultivation

Bacterial strain was inoculated in HS medium containing 2.0% D-glucose, 0.5% yeast extract, 0.5% peptone, 0.12% citric acid and 0.27% Na_2HPO_4 (%w/v) [21, 22] and modified HS medium prepared by replacing glucose with the carbon sources selected for our experiment, based on the literature. The carbon sources selected for the study were sucrose, fructose, glycerol, maltose, D-galactose, lactose, and glycine. The pH of all culture media was 6.0±0.2. Flasks were inoculated with 5% overnight grown bacterial culture and were incubated at 30°C±2°C for 7 days [19, 23]. Cell growth and BC yield from *A. aceti* cultivated in modified HS media supplemented with 2% (w/v) of different carbon sources were compared. At periodic intervals, 1 mL of culture broth was collected aseptically and was analyzed for BC yield and bacterial cell growth [24-26].

2.3 Recovery and purification of BC

BC was harvested from culture broth using an alkali treatment method [25]. Briefly, the culture broth was centrifuged at 5000 rpm for 15 min at room temperature to separate the pellet. The supernatant was discarded and the pellet was further subjected to alkali treatment using 1N NaOH solution in a 90°C water bath for 60 min to remove cell debris. The resulting solution was then centrifuged at 5000 rpm for 15-20 min, and the obtained pellet was washed with distilled water till its pH is neutral. The cellular pellet was then dried in a hot air oven at 60°C overnight [27].

The absorbance at a wavelength of 600 nm was measured in order to estimate the development of bacterial cells. The optical density of cell suspension was measured at 600 nm using a spectrophotometer (6850 UV/VIS Spectrophotometer JENWAY) to observe cell growth. Specific growth rate (μ) and doubling time (t_d) were calculated using equations 1 and 2 [28, 29]. For cell growth analysis, 100 µL of cell suspension was taken in 96-well plate and absorbance was read at 600 nm.

$$\mu = \frac{\ln N_t - \ln N_0}{(t - t_0)} \tag{1}$$

$$t_d = \frac{0.693}{\mu} \tag{2}$$

Where N_t and N_0, represent the number of bacterial cells during the exponential growth phase at time t and t_0, respectively, values which were used to identify the specific growth rate (μ) in equation 1. The specific growth rate (μ) signifies the rate of bacterial cell growth under the given culture conditions. [28, 29]. Equation 2 represents the relationship between specific growth rate and doubling time and was used to calculate the doubling time of bacteria cultivated in different carbon sources.

2.4 Physico-chemical characterization of bacterial cellulose

2.4.1 Characterization by Fourier Transform Infrared spectroscopy (FTIR)

BC samples produced using different carbon sources were characterized using FTIR spectroscopy (Perkin Elmer SPECTRUM BX II FT-IR System). FTIR spectra were recorded in transmittance mode over the 4000-400 cm^{-1} wavenumber range with 2 cm^{-1} intervals. Commercially available cellulose (Himedia, India), was used as the standard [28, 30]. Crystallinity ratios (Cr. R1 and Cr. R2) of BC were calculated using equations 3 and 4 as per Nelson and O'Connor [31].

$$\text{Cr. R1} = \frac{A_1}{A_{1'}} \tag{3}$$

$$\text{Cr. R2} = \frac{A_2}{A_{2'}} \tag{4}$$

Where A_1 and $A_{1'}$ correspond to the intensities of transmittance peaks at the wavenumbers 1,373 cm^{-1} and 2,900 cm^{-1}, and A_2 and $A_{2'}$ correspond to the intensities of transmittance peaks at the wavenumber 1,425 cm^{-1} and 895 cm^{-1}, respectively [15, 19].

### 2.4.2 Characterization by differential scanning calorimetry (DSC)

The thermal or heat properties of BC produced using different carbon sources were studied using a differential scanning calorimeter (Hitachi 700X) [32]. Approximately 5-7 mg of dried bacterial cellulose (BC) samples were used for the analysis. The BC samples were heated between 10 and 300°C at a heating rate of 10° per min using nitrogen gas (N_2). An empty aluminum pan was used as a reference for the analysis.

3. Results and Discussion

3.1 Effect of carbon source on bacterial cell growth and BC production

Bacterial cell growth and BC production by *A. aceti* MTCC 2623 were evaluated with HS medium supplemented with eight different carbon sources under static culture conditions for 7 days. Figure 1 represents the bacterial cell growth estimated as cell growth at 600 nm [33, 34]. The specific growth rate and doubling time of *A. aceti* were evaluated in HS medium supplemented with different carbon sources [28, 29]. The results demonstrated that all carbon sources supported the bacterial cell growth.

Table 1 shows the effect of carbon sources on specific growth rate and doubling time of *A. aceti* using different carbon sources under static culture conditions. The results showed that specific growth rate and doubling time varied with carbon source. HS medium supplemented with glucose resulted in the maximum specific growth rate (1.04 d^{-1}) and doubling time (0.67 d). Media supplemented with fructose showed lowest specific growth rate (0.29 d^{-1}) and doubling time (2.38 d). Cells show their maximum specific growth rate (μ) if the substrate is available in excess in the culture medium [35].
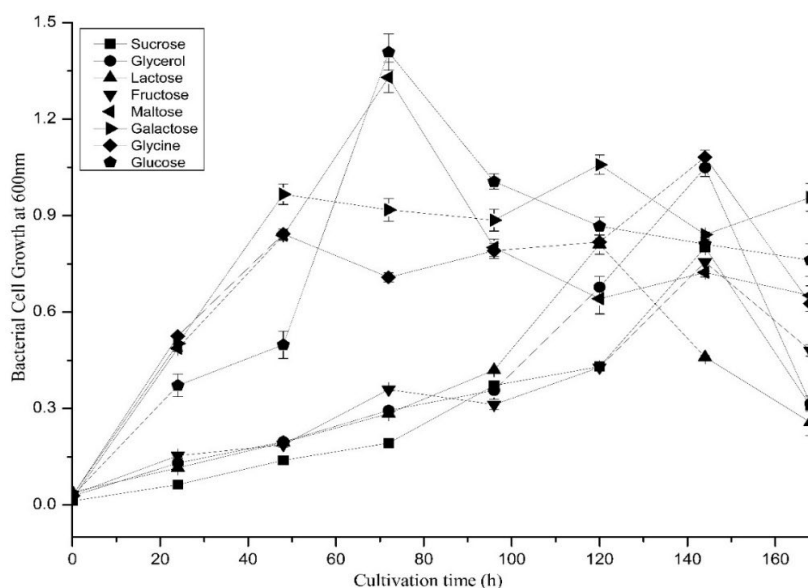
Figure-1. Bacterial cell growth of *A. aceti* MTCC 2623 with different carbon sources

Table 1. Variations in specific growth rate and doubling time of *A. aceti* MTCC 2623 using different carbon sources under static culture conditions

Carbon Sources	Sucrose	Glycerol	Lactose	Fructose	Maltose	Galactose	Glycine	Glucose
Specific growth rate(μ)	0.51	0.65	0.43	0.29	0.64	0.66	0.47	**1.04**
Doubling period (t_d)	1.36	1.07	1.59	2.38	1.08	1.06	1.47	**0.67**

BC was recovered as thin gelatinous sheet floating on the liquid-air interface after 5 days of incubation (Figure 2). The influence of different carbon sources on BC production was investigated and the results are shown in Figure 3. Figure 3 shows the BC production expressed as yield (g/L), calculated on a dry weight basis for the 8 carbon sources. The maximum amount of BC was obtained on day 5 in glucose supplemented HS medium (2.89±0.18 g/L) and the lowest BC yield (0.089±0.17 g/L) was observed with glycine as the carbon source. The results showed that the production of BC was affected by the types of carbon sources used. Cultivation with glycine resulted in the lowest BC yield. The maximum BC production was observed on day 5 [28, 36]. Our results are in accordance with those of Panesar *et al*. [37, 38], who reported a BC yield from *Acetobacter* sp. in the range of 1.5-1.73 g/L with HS media supplemented with glucose. Lin *et al*. [39] and Yim *et al*. [40] reported that HS medium was the standard recommended culture medium for BC production from different bacteria. Continuous research efforts have been attempted to optimize the culture media and bioprocess conditions for enhanced production of BC. Experimental evidences showed that using glucose as a carbon source enhanced BC yield as glucose is a precursor for cellulose synthesis. The ability of a bacterium to synthesize glucose from different carbon sources is known to affect its BC production abilities [41].
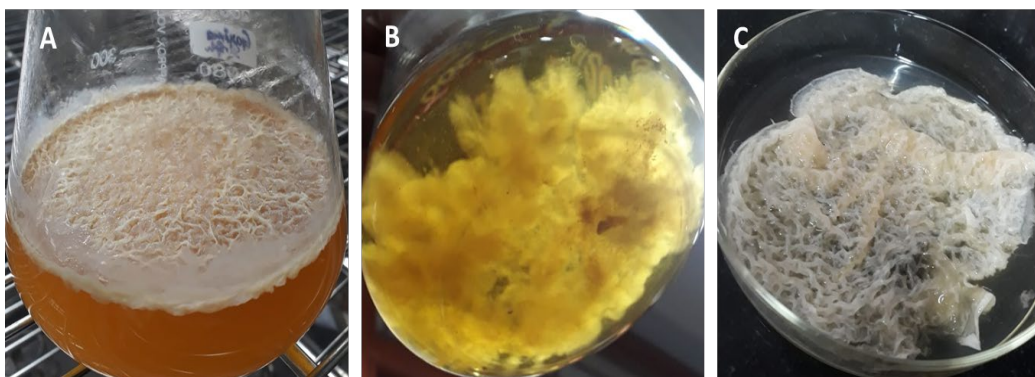
Figure 2. A and B, Production of BC from *A. aceti* MTCC 2623 on day 5, and C, Recovered BC film
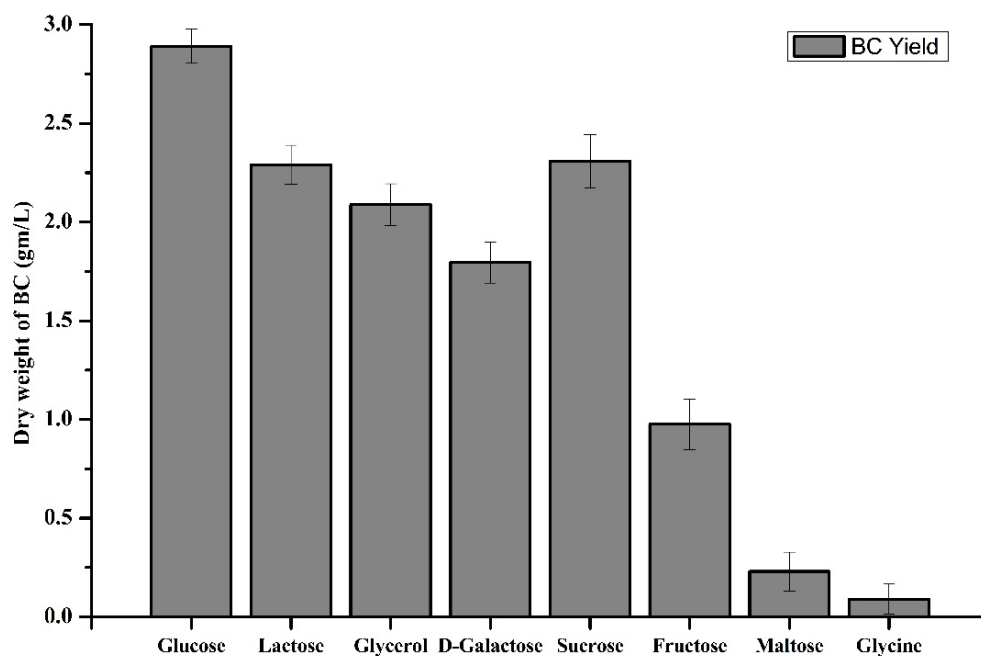
Figure 3. Effect of different carbon sources on BC production in HS medium using *A. aceti* MTCC 2623

3.2 FTIR analyses of commercial cellulose and BC produced by *A. aceti*

Figure 4 shows the FTIR spectra of BC samples produced by *A. aceti* from different carbon sources. Commercial cellulose was used as a reference [15, 30]. The intensities of infrared absorption peaks and their positions in FTIR investigations are typical of a particular compound. All FTIR spectra exhibited specific characteristic absorption bands in fingerprint regions, with slight variations implying the similar chemical structure of all BC samples produced from different carbon sources (Figure 4). All the 13 signature peaks assigned to specific functional group were tabulated in Table

2. The existence of absorption peaks in the wavenumber range of 3300-3200 cm^{-1}, assigned to O-H stretching, is an important characteristic of cellulose and the O-H stretching in that region is recognized as signature cellulose structural conformation [42].

The FTIR spectra of the BC samples displayed a number of distinctive absorption peaks. Several typical absorption peaks associated C-H stretching vibrational mode of CH$_2$ (methylene) and CH$_3$ (methyl) groups at nearly 2900 cm^{-1} [43] were observed (Table 2 and Figure 4). The H-O-H bending vibrational angle of absorbed water molecules at nearly 1650 cm^{-1}, CH$_2$ stretching vibrations at nearly 1426 cm^{-1}, and 1550 cm^{-1}, and vibrational mode for β-1, 4 glycosidic linkage, which is C-O-H antisymmetric stretching bridge at nearly 1160 cm^{-1}, were amongst others observed. C-H bending or out-of-phase specific molecular vibration of the CH$_2$ groups in the range of 1315 cm^{-1}, C-O twisting vibrations or C-C bonds of the monomer units as molecules linked together in the range of 1109 cm^{-1}, and 1050 cm^{-1}, the skeletal waves of the C-O-C pyranose rings or the C-O-H bending in polysaccharides resemble symmetric CH$_2$ group stretching or in-plane O-H link bending [44]. The unique functional groups and vibrations found in the cellulose and polysaccharide structures are represented by these absorption peaks. We noted other distinctive vibrations in the BC samples in addition to the previously mentioned absorptions. Stretching vibrational modes were identified as antisymmetric out of phase rings, and stretching β-1,4 glycosidic bonds amongst glucose units were observed around 870 cm^{-1} and 940 cm^{-1}. C-O bending peak at 1032 cm^{-1} and O-H out of phase bending at around 660 cm^{-1} was also observed [45].

Additionally, the absorption peaks in wavenumber range of 1450 cm^{-1}, 1380 cm^{-1}, 1109 cm^{-1}, 1050 cm^{-1}, and 1031 cm^{-1} can be assigned to the symmetric bending of the CH$_2$, the C-C bonds in polysaccharide monomers, the deformation of the C-H molecule, and the skeletal shifting of the C-O-C pyranose ring [45], respectively. Our findings suggested that BC produced by *A. aceti* MTCC 2623 consisted mostly of cellulose I (assigned to absorption peaks at around wavenumber 3300, 1430, 1160 and 950 cm^{-1}), with little presence of cellulose II, which is indicated by absorption peaks at around 1322 cm^{-1} [30], as shown in Table 2. Cellulose is known to exist in 2 allomorphic forms in nature: cellulose I and II, with cellulose I being dominant [46]. The intensities of absorption bands at 1375 cm^{-1}, 2900 cm^{-1}, 1500 cm^{-1} and 883 cm^{-1} were measured and used to calculate crystallinity index by Nelson and O' Connor equation [31] as shown in Table 3. Commercial cellulose showed lowest CR. R1, value as compared to BC sample produced from various carbon sources. Data obtained from crystallinity ratios suggested that all BC samples had higher crystallinity than commercial cellulose. The absence of non-characteristic peaks in the BC samples compared to commercial cellulose suggested that the BC samples were of higher purity. Therefore, the applicability of BC in various fields is suggested [47]. Similar findings were also observed for BC samples produced by *G. xylinus* ATCC 324 using different carbon sources [48].

3.3 Differential scanning calorimetry (DSC) analysis

Figure 5 shows a graph of thermal stability obtained by the differential scanning colorimetry method (DSC). Stumpf *et al.* [49] reported that the investigation on thermal properties of BC might be significantly important for their industrial applications and utilization. DSC analysis of the thermal stability of BC samples produced from different carbon sources was used to determine the glass transition temperature (Tg) of BC samples and commercial cellulose over the temperature range of 50°C-250°C (Figure 5). From the curve patters shown in Figure 5, transformations related to loss of water due to evaporation appeared as endothermic peaks During the initial stages of thermal treatment, all BC samples exhibited endothermic peaks that appeared in the temperature range of 90-120°C [50]. This may be attributed to the transformation related to loss of water (moisture content) due to evaporation.
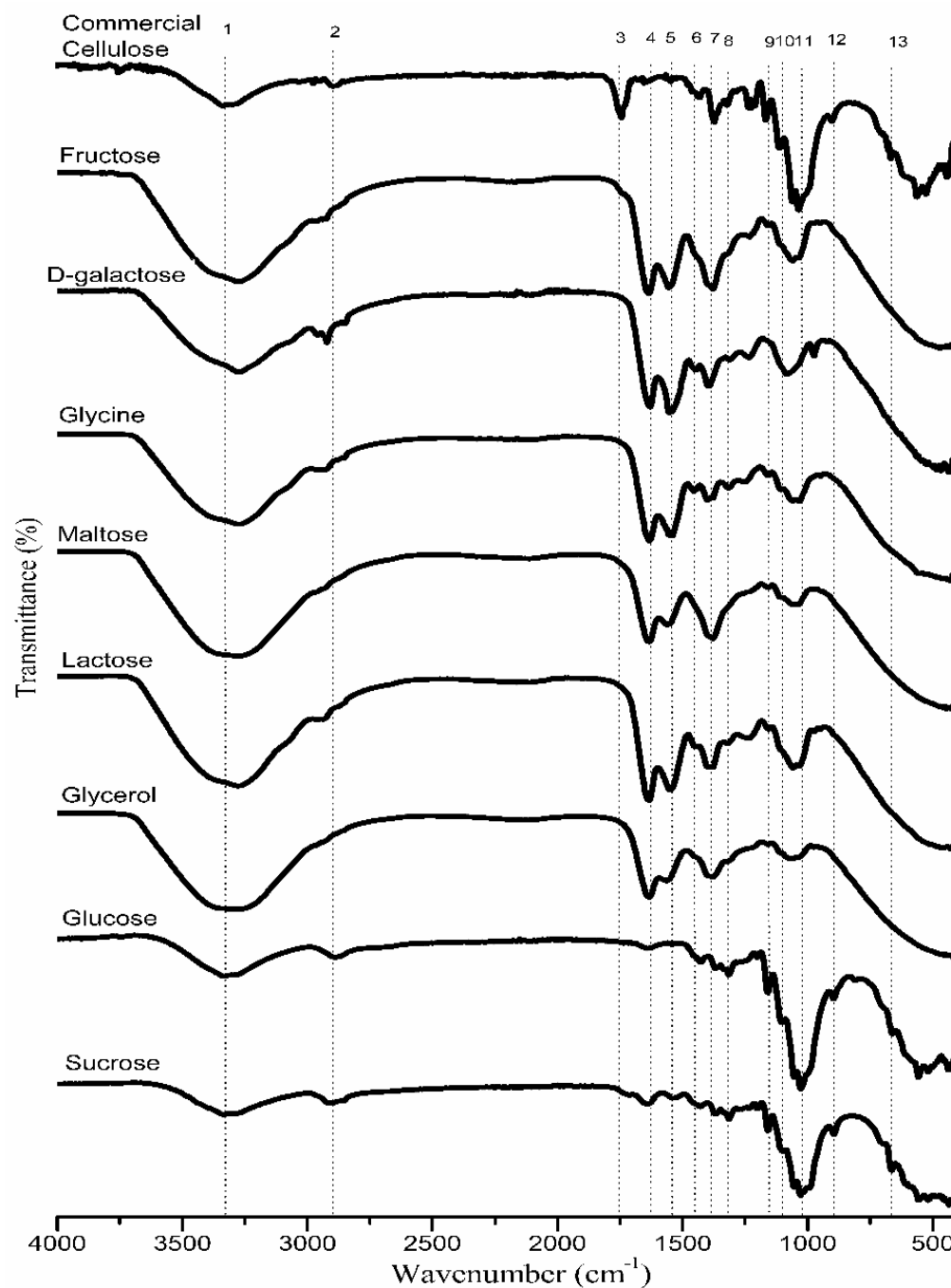
Figure 4. Comparative analysis of FTIR of commercial cellulose and BC produced by *A.aceti* MTCC 2623 from different carbon sources

Table.2. Analysis of characteristic IR absorption peaks corresponding to functional groups of BC synthesized using *A. aceti* MTCC 2623 from different carbon source

Peak Number	Bond/ Functional Group	Wavenumber cm^{-1}								
		Commercial	Sucrose	Maltose	Lactose	Glycine	Glycerol	Glucose	Galactose	Fructose
1	OH, stretching vibration	3340	3265	3284	3283	3293	3286	3334	3293	3345
2	C–H stretching of CH_2 and CH_3 groups	2903	2970	2923	2962	2959	2940	2887	2940	2919
3	H–O–H bending form of absorption	1680	1635	1632	1634	1636	1635	1647	1640	1647
4	CH_2 stretching vibration	1530	1558	1556	1549	1566	1551	1429	1564	1540
5	CH binding	1367	1381	1397	1403	1383	1383	1370	1383	1372
6	O–H in-sphere bending	1317	1322	1314	1319	1317	1322	1315	-	1316
7	C–O–C asymmetrical stretching vibrational of 1,4-β-D-glycosidic linkage	1162	1160	-	1159	1163	1159	1161	1171	1160
8	C–O bending vibration	1109	1109	-	1110	-	-	1104	1114	1100

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Table.2. Analysis of characteristic IR absorption peaks corresponding to functional groups of BC synthesized using *A. aceti* MTCC 2623 from different carbon source (continued)

Peak Number	Bond/ Functional Group	Wavenumber cm^{-1}								
		Commercial	Sucrose	Maltose	Lactose	Glycine	Glycerol	Glucose	Galactose	Fructose
9	C–O–C vibration of the pyranose ring	1055	1065	1086	1062	1061	1070	1054	1064	1055
10	C-H binding vibration	1032	-	-	1032	1037	-	1030		1029
11	β-1,4 glycosidic bonds amongst the sugar units	898	948	977	952	979	952	897	878	897
12	O–H out-of-step bending vibration	662	-	-	-	-	-	661	-	665
13	C-O-C stretching	557	-	-	-	-	-	558	-	558

Table 3. Crystallinity ratios (Cr. R) of commercial and BC samples produced from different carbon sources at different fingerprint regions of FTIR spectrum

Cellulose Samples	Commercial Cellulose	Sucrose	Maltose	Lactose	Glycine	Glycerol	Glucose	Galactose	Fructose
Cr. R$_1$	0.52	1.37	1.45	1.23	0.97	1.18	0.44	1.06	0.43
Cr. R$_2$	2.13	3.06	2.16	2.11	0.64	2.57	1.27	2.75	1.42
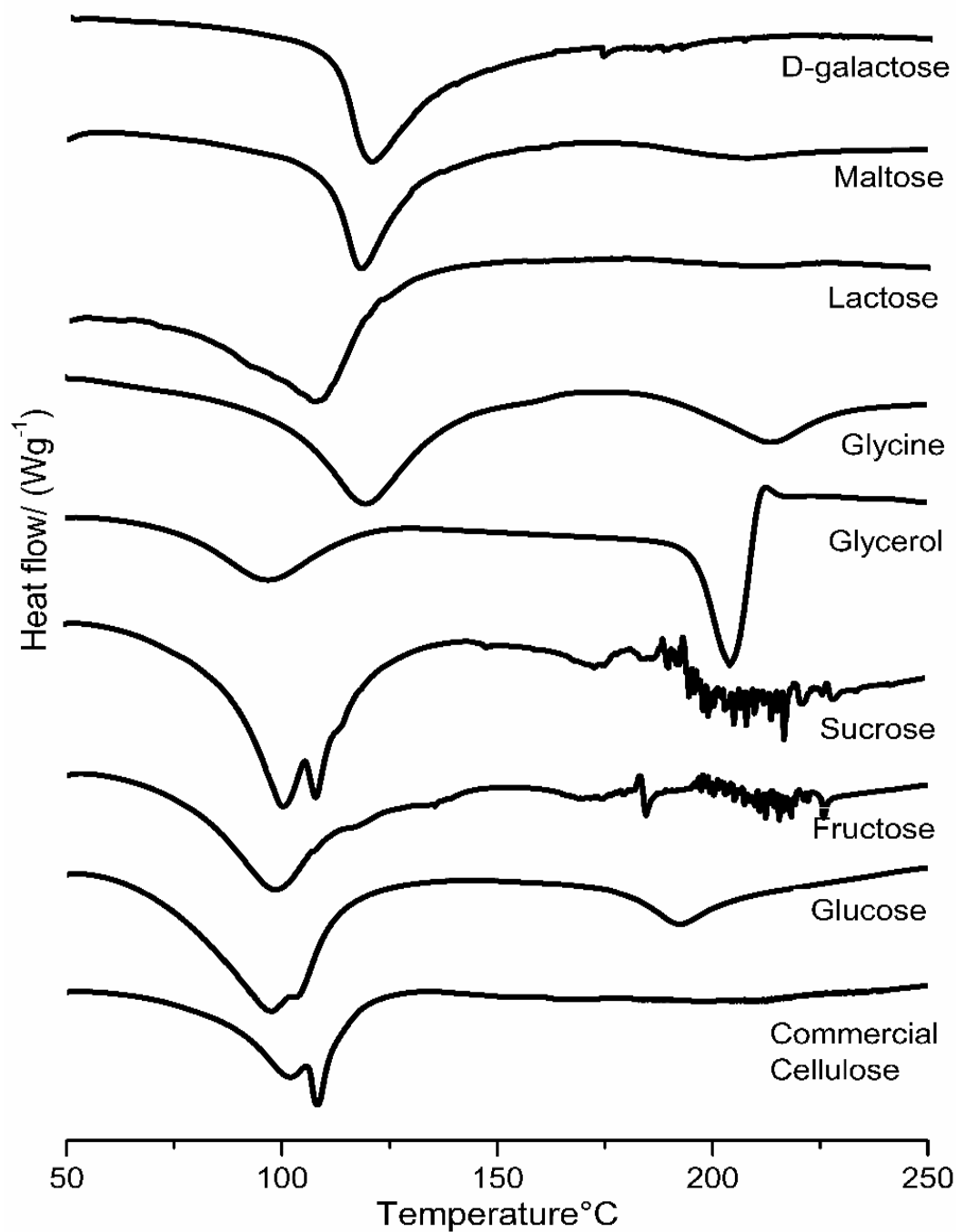
Figure 5. Comparative differential scanning calorimetry (DSC) analysis of the commercial cellulose and BC produced from different carbon sources by *A.aceti* MTCC2623 grown in the HS media

Previous research findings showed similar transformation over the 80°C-140°C range that were related to the evaporation of water and melting of crystalline phase of cellulose [51, 52]. Another endothermic peak (200-250°C) that was observed in BC samples produced from glucose, fructose, sucrose, glycine, glycerol, may be associated with various types of decomposition of the BC sample, including depolymerization or decomposition of glucose residues in cellulose chains [53]. Determination of glass transition temperature and decomposition of BC might be useful for determining thermal stability. Researchers have shown that the thermal degradation behavior of a polymer is associated with changes in structural parameters such as crystallinity, monomer orientation of the fibers and its molecular weight [32, 53]

4. Conclusions

In this study, the production of BC by *A. aceti* MTCC 2623 was investigated using 8 different carbon sources (sucrose, fructose, glycerol, maltose, d-galactose, lactose, glycine and glucose) under static culture conditions for 7 days. Supplementing HS medium with 2 % (w/v) glucose resulted in the highest BC yield (2.89±0.18 g/L) whereas supplementation with glycine resulted in the lowest BC yield (0.089±0.17 g/L). The results showed that the specific growth rate and doubling time of *A. aceti* varied with the type of carbon source used. Furthermore, we investigated the effect of carbon sources on the physicochemical characteristics of BC. FTIR analysis showed that BC samples were pure and exhibited no significant variations in the fingerprint regions. BC samples were crystalline in nature, compared to commercial cellulose. DSC analysis showed that BC produced using glucose showed improved thermal stability compared to commercial cellulose. Our findings showed that the types of carbon source affected the microbial growth kinetics and cellulose production profile of BC from *A. aceti*. However, no significant variations were observed in the characteristics of BC produced. The study may be useful for optimizing the culture medium for enhanced BC production and its proposed industrial and biomedical applications.

5. Acknowledgements

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