STM JOURNALS

Research & Reviews: A Journal of
Biotechnology

https://journals.stmjournals.com/rrjobt/

ISSN: 2231-3826 (Online)
ISSN: 2347-7245 (Print)
Volume 14, Issue 2, 2024
May–August
DOI (Journal): 10.37591/RRJoBT

Research RRJoBT

Influence of Agitation and Static Cultivation on Physicochemical Characteristics of Bacterial Cellulose Produced by *Gluconacetobacter liquefaciens* MTCC 3135

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Abstract

Bacterial cellulose (BC) a with applications spanning biomedical, food, paper, electronics, and diverse industrial processes. Its unique properties include high purity, biocompatibility, and flexibility. BC is a versatile natural biopolymeric material, secreted by certain acetic acid bacteria. BC has numerous benefits over plant cellulose due to distinctive features, e.g., higher polymerization degree and purity, superior crystalline structure, water retention, biocompatibility, and biodegradability, which make BC a valuable material in creating sustainable and innovative solutions across various industries. However, the high operating cost, expensive culture media components, and low productivity often limit its widespread industrial usage. In our study, BC production by Gluconacetobacter liquefaciens MTCC 3135 was analyzed under agitation and static cultivation in the Hestrin–Shramm (HS) medium. Variations were observed in microbial growth kinetics parameters of G. liquefaciens under agitation and static cultivation. The highest BC yield at 3.55 ± 0.26 g/L was obtained in static cultivation, while agitated culture condition yielded the lowest BC at 2.59 ± 0.16 g/L. BC samples produced under agitation and static culture were purified using NaOH treatment and were subjected to physicochemical characterization using FTIR, XRD, and DSC. FTIR spectra showed peak shifting and variations in peak intensities for BC samples produced under agitation and static culture when compared to commercial cellulose (HiMedia, India). BC produced under static culture was more crystalline compared to BC samples produced under agitation, as determined by FTIR and XRD. The research contributes valuable insights into alternative sources of BC production, targeting to fill gaps in knowledge and promote sustainable cellulose production.

Keywords: Bacterial cellulose, *Gluconacetobacter liquefaciens*, static, agitation, FTIR, XRD, DSC

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Received Date: March 28, 2024
Accepted Date: April 08, 2024
Published Date: May 10, 2024

Citation: Garima Singh, Pammi Gauba, Garima Mathur. Influence of Agitation and Static Cultivation on Physicochemical Characteristics of Bacterial Cellulose Produced by *Gluconacetobacter liquefaciens* MTCC 3135. Research & Reviews: A Journal of Biotechnology. 2024; 14(2): 1–11p.

INTRODUCTION

Cellulose is a natural biopolymer that is abundant in nature. It is a major part of the cell wall in vascular plants, where it is chemically linked to other components such as hemicellulose, lignin, and several other compounds. Structurally, it is made up of glucose monomers connected via β-(1,4) glycosidic linkages [1, 2]. There is a growing demand for cellulose, which is expected to be driven by factors such as changing consumer preferences among emerging economies, the demand for sustainability, and global industrial expansion [3]. This would encourage the expansion of the global cellulose market [4]. The global cellulose market which was valued at US$ 40.56 billion in 2022 is expected to reach up to US$ 93.10 billion by 2030,

with a projected CAGR of 9.67% during 2023–2030 [5]. With the increased demand for renewable and eco-friendly biomaterials, manufacturers are seeking sustainable alternatives to petrochemical-derived polymeric materials. The increasing demand for cellulose and its derivatives adversely affects the ecosystem, leading to environmental damage [3, 4].

BC is considered a polymeric material that is produced extracellularly by several bacterial genera such as *Acetobacter, Azotobacter, Salmonella, Sarcina*, and *Gluconacetobacter*. The most extensively studied BC producer is *Acetobacter xylinum* which was isolated more than 200 years ago from Nata de Coco in the Philippines [6]. Extracting cellulose from plant sources using a conventional approach includes alkali-bleaching treatment at high temperatures, which results in serious environmental issues. To explore new sustainable biomaterials, bacterial cellulose (BC) is a promising alternative, owing to its superior physicochemical properties compared to plant cellulose [7, 8]. BC has a nanofibrillar three-dimensional network structure stabilized by extensive hydrogen bonding. BC exhibits higher purity, higher degree of polymerization, improved crystallinity and thermal stability, biocompatibility, good moisture retention, and improved mechanical strength, making it versatile for various industrial and biomedical applications, notably in cellulose-based textiles, BC-based electroconductive composites, BC-based functional foods and nutraceuticals, and cosmeceuticals as nanocomposite films in OLED [9, 10]. In recent years, there has been an increased focus on xeno-free biomaterials in the healthcare sector owing to regulatory and ethical concerns. BC serves as an excellent biomaterial in wound dressing, artificial blood vessels, vascular grafts, and drug and protein delivery systems because of its animal-free and human-free origin. BC scaffolds have proven effective in tissue engineering and wound healing, demonstrating their potential for diverse applications [11]. Despite its promising attributes, BC production faces challenges, such as low yield and high cost of production. Some factors affecting BC production include the choice of microorganisms, carbon and nitrogen sources, nutrient medium composition, and cultivation methods. Researchers have actively addressed these issues through extensive research on enhanced BC production from cellulose-producing bacteria isolated from rotten fruits and vegetables [12, 13]. Media optimization approaches based on utilizing agro-industrial residues as carbon and nitrogen sources offer economic advantages. Several studies have focused on utilizing agro-industrial byproducts, such as sugar and starch, fruit and vegetable peels, rice milling waste, and several other agricultural processing wastes as alternative feedstocks for cost-effective BC production [12, 14]. Innovative approaches, such as using elephant grass acid hydrolysate and distillery wastewater from the winery industry, have shown increased yields and cost-effectiveness compared with conventional substrates [15, 16].

In this study, we explored the potential of *Gluconacetobacter liquefaciens* MTCC 3135 for BC production as an alternative to *Acetobacter* strains in an HS medium under agitation and static cultivation. The study had the following objectives: determination of bacterial cell growth kinetics, evaluation of BC yield, and physicochemical characterization using FTIR, XRD, and DSC. To the best of our knowledge, this is the first report highlighting the effect of the cultivation method on cell growth kinetics, BC yield, and physicochemical characteristics of BC.

MATERIALS AND METHODS

Microorganisms, Media Composition, and Cultivation

A native bacterial strain, *Gluconacetobacter liquefaciens* (MTCC 3135), was obtained from MTCC (India). For culture revival, Hestrin-Schramm (HS) medium containing 20 g/L glucose, 5 g/L peptone, 5 g/L yeast extract, 2.7 g/L Na_2HPO_4, and 1.2 g/L citric acid monohydrate, pH 6.0 at 30 ± 2°C was used [17]. The purity of the recovered bacterial cultures was tested using Gram staining. For long-term storage, glycerol stocks (20%) were prepared and were stored at -80°C [18]. Bacterial cell suspensions (0.1 ml) were inoculated in 20 mL of HS broth for culture revival. Active bacterial cultures were maintained on HS agar plates. For primary inoculum preparation, bacterial colonies from pure culture plates were inoculated into 50 ml of sterilized HS broth [19]. Microbial growth and BC yield were studied for five days in HS medium, followed by incubation at 30 ± 2°C under agitation and static

cultivation. At regular intervals, 1 ml sample was collected from the respective flasks and used for further analysis [20]. All the experiments were conducted under sterile conditions to maintain the integrity of the results.

Purification and Harvest of BC

After fermentation, the bacterial cellulose (BC) membranes were carefully separated from the culture medium and washed repeatedly with distilled water. Recovered membranes were treated with 1N NaOH in a hot water bath (90°C) for 60 min to remove any bacterial cells and other impurities adhering to the BC membranes [21]. After alkali treatment, the membranes were rinsed several times with distilled water to eliminate the alkali until the pH became neutral [22]. The recovered purified cellulose membranes were air-dried overnight at 60°C until the weight was constant. The BC production was estimated to be dry wt. for BC, expressed in g/l.

Estimation of Bacterial Cell Growth

Cell broth samples were treated with cellulase enzyme (1U) before the growth studies. Microbial growth was then determined by measuring the optical density at 600 nm [23] and was demonstrated as the specific growth rate (μ) and doubling time (td) using equations 1 and 2 [24, 25].

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

Where, N_t and N_0 correspond to the cell counts measured at t and t_0, and μ represents the specific growth rate of *G. liquefaciens* calculated under agitation and static cultivation. The doubling time t_d of the bacteria was determined using the following formula [24, 25]:

$$t_d = \frac{0.693}{\mu} \quad (2)$$

Determination of Physicochemical Characteristics of BC

FTIR Analysis of BC

The purified dried BC membranes were cut to a size of 2 mm × 2 mm for FTIR analysis using Fourier transform infrared (FTIR) spectroscopy (Spectrum BX-II spectrophotometer, Perkin Elmer). The samples were scanned over a wavenumber range of 4000−400 cm^{-1} with 2 cm^{-1} resolution [26, 27]. Commercial cellulose (HiMedia, India) was used as a reference.

The crystallinity index of the BC samples was calculated [28], using equations 3 and 4:

$$\text{Cr. R1} = \frac{A_1}{A_{1'}}, \quad (3)$$

$$\text{Cr. R1} = \frac{A_2}{A_{2'}} \quad (4)$$

Here, A_1 and A_1' represent the peak intensities at 1,372 cm^{-1} and 2,900 cm^{-1}, respectively, and A_2 and A_2' represent the band intensities at wavenumber 1,425 cm^{-1} and 895 cm^{-1} respectively.

X-ray Diffraction Studies of BC

BC samples were subjected to X-ray diffraction analysis using the modified method of [29] on a diffractometer (Shimadzu 6000 XRD, Japan) with Cu Kα radiation at a voltage of 40 kV, current of 40 mA, and scattering angle of 2θ =20° to 70° [25, 30].

The crystallinity percentage of the cellulose samples was determined using the Segal equation Eq.5. [31].

$$Cr.I\ (\%) = \frac{I_{200} - I_{am}}{I_{200}} \text{ X } 100$$

Where, Cl is the crystallinity percentage, I_{002} is the intensity maxima of the (002) lattice diffraction, and I_{am} is the intensity of the diffraction.

Thermal Stability Analysis Using DSC

BC samples were subjected to DSC analysis to evaluate their thermal stability on a Hitachi DSC thermal analyzer 700X [32] using 3 mg of the sample. Samples were scanned over a temperature range of 0°C–300°C and a heating rate of 10°C min^{-1} using an aluminum pan and nitrogen with a flow rate of 70 mL min^{-1} to prevent sample oxidation [33].

RESULT AND DISCUSSION

Determination of Bacterial Growth Kinetics of *G. liquefaciens* MTCC 3135

G. liquefaciens MTCC 3135, used as a native strain in our study, is a gram-negative, rod-shaped, obligate aerobic bacterium (Figure 1a). *Gluconacetobacter sp.* has been previously reported for BC production [23]. However, a detailed analysis of the role of agitation and static cultivation on microbial growth, BC production, and physicochemical characteristics has not yet been reported. The influence of the cultivation method on microbial growth of the native strain *G. liquefaciens* MTCC 3135 was expressed as the specific growth rate and doubling time (Table 1) [34]. Incubation under static conditions resulted in a lower doubling time (0.89 d) and a high specific growth rate (0.78 d^{-1}) compared to agitated conditions with a doubling time of 1.18 d and a specific growth rate of 1.37 d^{-1}. Previous studies have suggested that microbial growth rate is affected by nutrient availability, and cells exhibit a higher specific growth rate when the culture medium exceeds substrate availability [25].

Table 1. Microbial growth kinetics of *G. liquefaciens* MTCC 3135 in HS medium under different cultivation methods.

Culture condition	Specific growth rate (μ)	Doubling time (t_d)
Static	0.78	0.89
Agitated	0.59	1.18

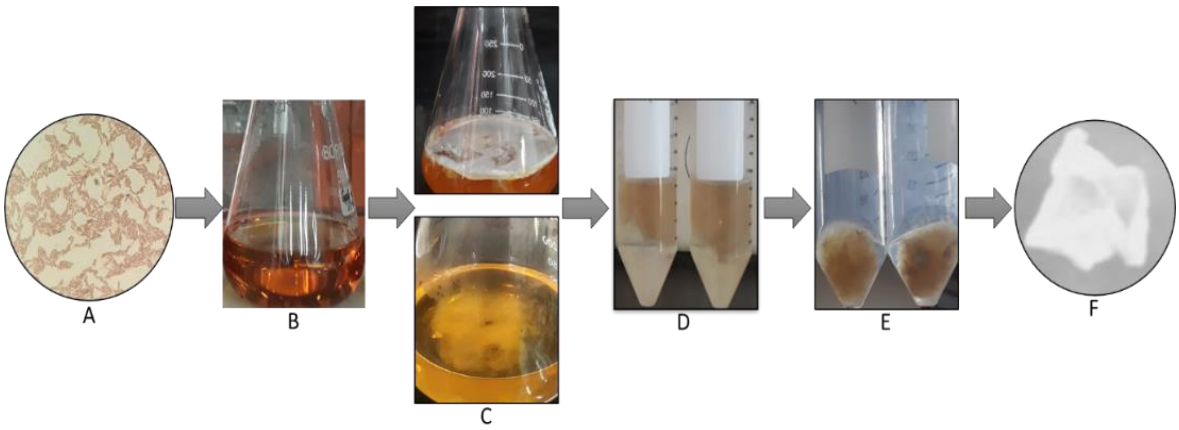

Figure 1. Production and purification of BC. (A) Bacterial morphology, (B) HS media, (C) production of BC film, (D&E) purification of BC, and (F) dried cellulose.

Cultivation Method and BC Yield

BC produced by *G. liquefaciens* MTCC 3135 under agitated and static conditions exhibited morphological variations. BC was observed as a thin gelatinous matt on the medium surface under static cultivation, whereas agitation resulted in irregular sphere-like pellicles, as shown in Figure 1c. Shear forces generated during agitation affect cell viability, resulting in the generation of irregular BC pellicles [22, 35]. The synthesis of BC under static cultivation is modulated by carbon supply and aeration of the medium, resulting in increased cell growth and stronger C-H bonding [36]. BC production, calculated as dry weight of BC per liter of culture medium, was higher at 3.55 ± 0.26 g/L in static conditions,

compared to agitated conditions calculated as 2.59 ± 0.16 g/L, as shown in Figure 2. Studies have shown that agitated conditions induce the production of various non-cellulosic-producing mutants, which have a detrimental effect on BC production [37], resulting in lower cellulose yields in comparison to static cultivation [38].

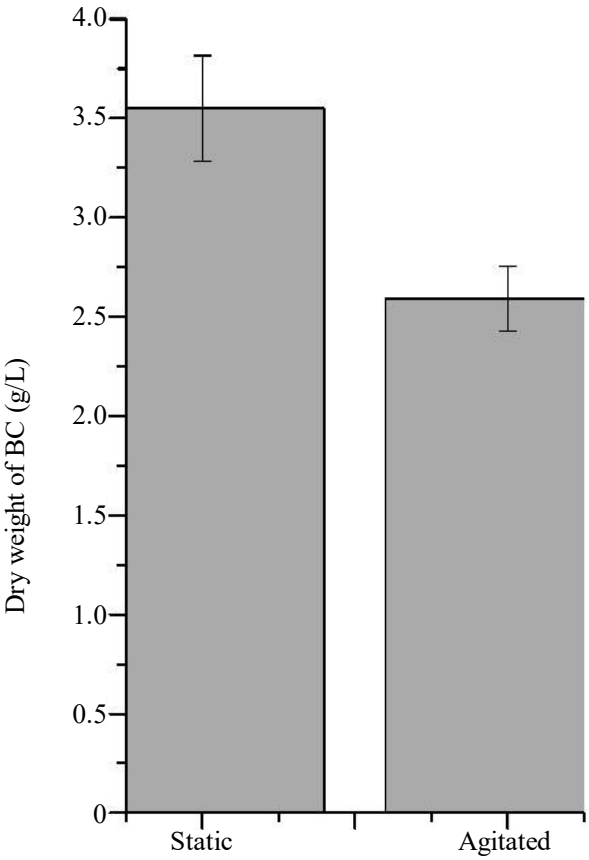

Figure 2. Production of BC from *G. liquefaciens* MTCC 3135 in HS medium agitation and static culture condition.

Characterization of BC by FTIR

FTIR analysis was carried out to analyze the chemical bonding present in the bacterial cellulose produced in the HS medium under agitation and static cultivation. The IR spectra of commercial cellulose (HiMedia, India) and BC were obtained in the transmittance mode in the wavenumber region (4000–400) cm^{-1} [27, 28]. The infrared (IR) spectra of bacterial cellulose and commercial cellulose revealed the presence of characteristic absorption bands in the fingerprinting region for both commercial and bacterial cellulose samples, as shown in Figure 3.

The bands at 2876, 2138, and 2921 cm^{-1} were assigned to the C-H stretching vibration in polysaccharides. Peaks at 1645 cm^{-1}, 1580 cm^{-1}, and 1596 cm^{-1} refer to the vibrations of water molecules [39, 40]. The signature fingerprint absorption peaks of cellulose were observed at wavenumbers 1314, 1259, 1131, 906, and 557 cm^{-1}) in commercial cellulose & (1378–1396, 1162, 1023 cm, 932 cm, 850, and 427 cm^{-1}) assigned to the stretching and bending vibrations of -CH_2 and -CH, -OH, and -COC bonds in BC [41]. Peaks at 1375, 2900, 1500, and 883 cm^{-1} were selected to calculate the crystallinity index, Cr. R1= I_{1375}/I_{2900} or Cr. R2 = I_{1500}/I_{883} [28, 29], as shown in Table 2.

The presence of an intense band at 3500–3000 cm^{-1} corresponds to the stretching vibration of the O-H and C-H bonds in polysaccharides. Our BC samples revealed the presence of sharp broad peaks at wavenumber 3334 cm^{-1} and 3277 cm^{-1}, confirming the purity of the samples [25].

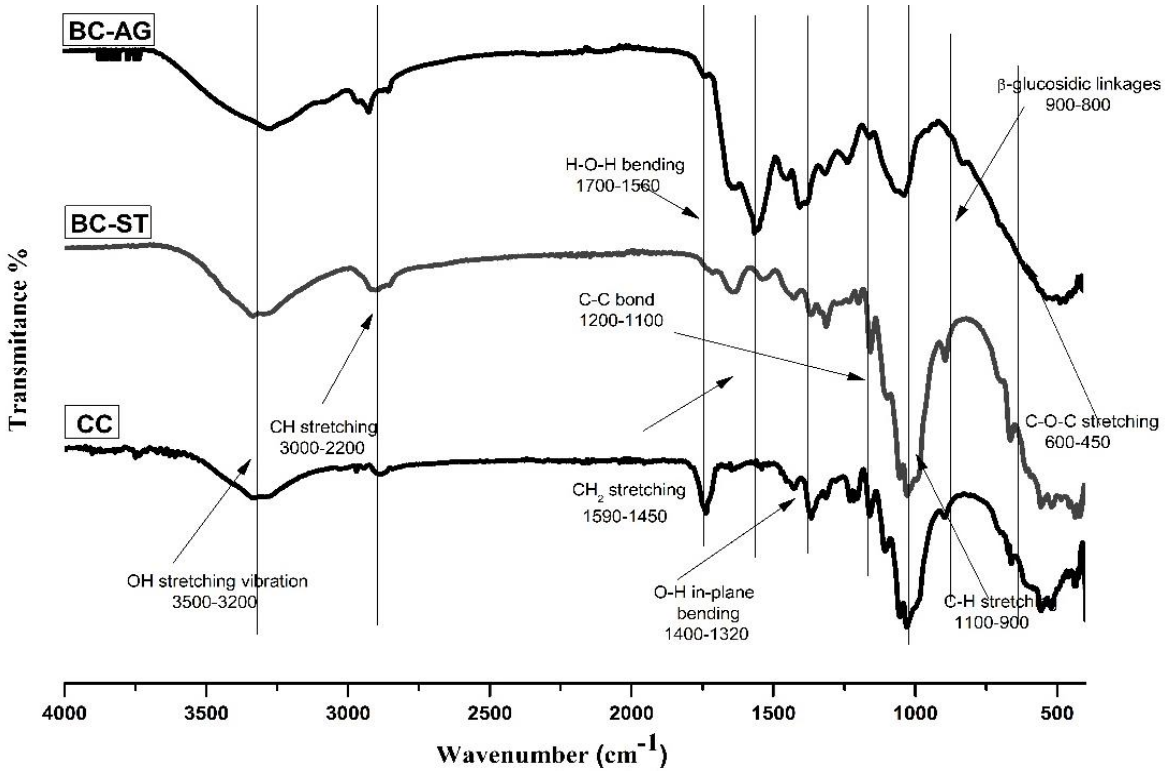

Figure 3. FTIR spectrum of commercial cellulose and BC produced by *G. liquefaciens* MTCC 3135.

Table 2. FT-IR crystallinity index of BC produced by *G. liquefaciens* MTCC 3135.

Sample	Band intensity (absorbance) at wavenumber cm^{-1}				Cr. R1 I_{1375}/I_{2900}	Cr. R2 I_{1500}/I_{883}
	1375	*2900*	*1500*	*883*		
Commercial Cellulose	0.022 (1354)	0.017 (2886)	0.010 (1530)	0.038 (890)	1.57	0.91
Static-BC	0.183 (1379)	0.072 (2921)	0.184 (1515)	0.169 (853)	2.50	1.29
Agitated-BC	0.027 (1370)	0.030 (2880)	0.015 (1495)	0.012 (837)	1.28	0.87

X-ray Diffraction Studies of BC

X-ray diffraction patterns of the commercial cellulose and BC samples produced by *G. liquefaciens* under static culture conditions are summarized in Figure 4. The diffraction patterns of the BC samples exhibited broad diffraction peaks at 2Θ 14.54° and 22.4°, which correspond to the (110) and (200) crystal planes of the two cellulose allomorphs 1α and 1β [42, 43]. Our results showed the presence of similar diffraction peaks at 2Θ 14.54° and 22.4°, with higher intensity for the BC samples compared to commercial cellulose (Figure 4), which also showed that the BC samples primarily exhibited a cellulose 1α pattern [44]. The crystallinity index was determined using the Segal equation [31]. BC samples produced under static conditions exhibited higher crystallinity (81.7%) than commercial cellulose (72%), whereas BC produced under agitated culture conditions was less crystalline (68.4%) (Table 3).

Analysis of Thermal Behavior Using DSC

DSC analysis determines the amount of heat absorbed or released by any material with respect to temperature change [45]. Figure 5 shows the DSC thermograms for commercial cellulose and bacterial cellulose produced by *G. liquefaciens* MTCC 3135 in HS medium under static culture conditions. DSC patterns showed the initial degradation phase in the temperature range 60–150°C for BC, appearing as an endothermic peak [44, 46], attributed to dehydration of surface water, and transformational changes attributed to crystalline phase melting of cellulose [47]. An endothermic peak around 100°C was observed for commercial cellulose owing to water loss. Samples showed water loss temperature of 98.10°C and an enthalpy of approximately 60.78 J g^{-1} [48].

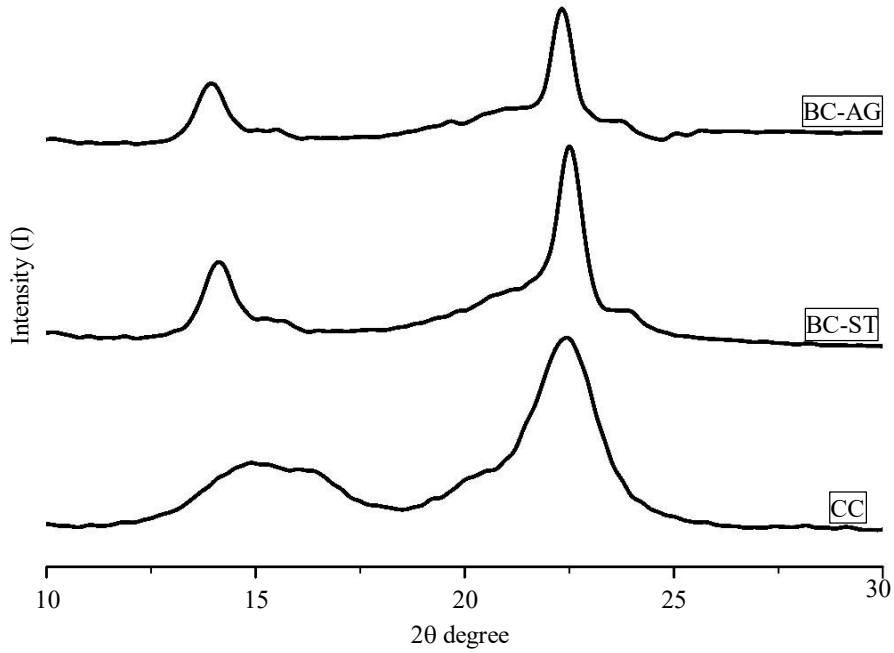

Figure 4. XRD diagrams of commercial cellulose and BC produced by *G. liquefaciens* under agitation and static conditions in the HS media.

Table 3. Crystallinity index of commercial cellulose and BC samples.

Samples	**Cr. I %**
Commercial cellulose	72%
BC-Static	81.7%
BC-Agitated	68.4%

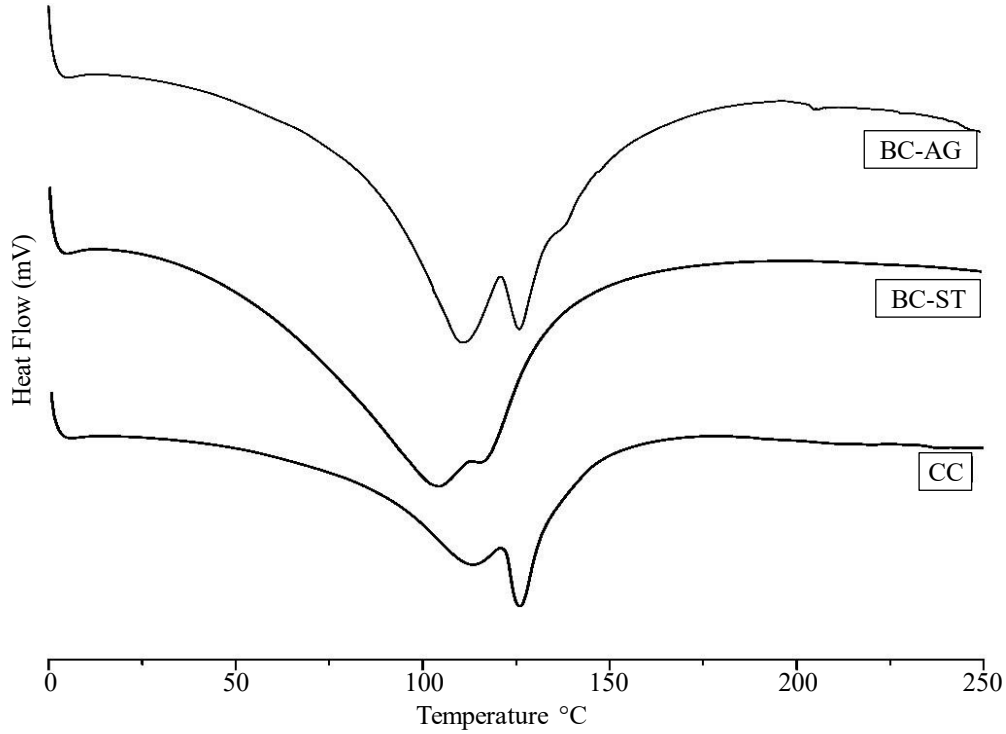

Figure 5. Comparative Differential Scanning Calorimetry (DSC) analysis of the commercial cellulose and BC produced by *G. liquefaciens* in the HS media.

CONCLUSION

This study summarizes the production of BC from *G. liquefaciens* MTCC 3135 under agitation and static cultivation conditions to evaluate microbial growth, BC yield, and physicochemical characteristics. Culturing bacteria under agitation conditions affects cell growth and results in a lower BC yield. FTIR analysis revealed the purity of BC samples exhibiting mainly cellulose 1α allomorphs, with high crystallinity compared to commercial cellulose. The thermal stabilities of BC and commercial cellulose exhibited no significant variation. The XRD patterns showed the presence of two characteristic diffraction peaks, indicating the crystalline nature of the BC produced by *G. liquefaciens. The* study opens new avenues for exploring *G. liquefaciens* MTCC 3135 as an efficient BC producer for widespread applications.

Acknowledgments

The authors are thankful to the Council of Science and Technology, Uttar Pradesh (UPCST), for funding this research work, and the Department of Biotechnology and Department of Physics and Materials Science and Engineering, Jaypee Institute of Information Technology Noida, India, for providing the necessary facilities for this work.

Authors Contribution

All authors made substantial contributions to the conception and design of the experiments, data acquisition, analysis, and interpretation of data, drafting the manuscript and critical revision for intellectual content, agreed to submit to the current journal, gave final approval of the version to be published, and agreed to be accountable for all aspects of the work.

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