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Optimization, Characterization, and Cytotoxic Study of Bio Cellulose by *Acetobacter sp* Strains to Engender Biodegradable Food Wrapper

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Abstract

Acetic acid bacteria synthesized microbial cellulose were isolated from various citrus fruits, enabled by increased cellulose production to develop a biodegradable polymer as a food wrapper. The objective of the research cynosures on the isolation, enrichment, identification, and optimization of bacteria that produce cellulose, characterization, and cytotoxic study of the obtained cellulose. Two highly effective cellulose producers, *Acetobacter lovaniensis* (A1) and *Acetobacter fabarum* (A2), were isolated based on their morphology, biochemical analysis, and 16s rRNA sequencing. Studies were conducted to optimize pH, temperature, inoculum size, nitrogen, and carbon sources. Strain A1 produced 0.715 g/100 ml, whereas A2 produced 0.856 g/100 ml of cellulose under optimum growth conditions. The characteristics of microbial cellulose were examined using scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FTIR). A cytotoxicity study for the obtained cellulose has been conducted with mouse embryo fibroblast cells (3T3-L1) and showed 97% viability of cells with the lowest concentration of 12.5 µg/ml. These isolates could be employed in fermentation technology to produce cellulose polymer-based sustainable biodegradable food wrappers.

Keywords: Acetic Acid Bacteria, Cellulose, SEM, FTIR, 16s rRNA, Cytotoxicity

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INTRODUCTION

Microbial polymers are a range of macromolecules, including polysaccharides, polyesters, polyamide, and inorganic polyanhydrides. Bacterial polymers are renewable resources that gain the wide limelight in recent times for their application in textile, paper making, packaging, and biomedical application, and aid in numerous pollution problems that are of environmental and economic concerns.[1-3] Cellulose is one of the polysaccharides made up of glucose units linked together by beta-(1-4)- glycosidic linkages, considered to be an inexhaustible unique material source with many applications.[4] Though plants are the primary cellulose producers, bacterial cellulose producers possess different physicochemical properties from those of plants.[5]

The production of cellulose by bacterial cultures is an engrossing method to obtain pure cellulose by the selection of substrate, strain selected and its condition, cultivation method, temperature, pH, inoculum ratio, and additives.[6] Bacterial cellulose is one of the classes of nano celluloses. Compared to other sources of cellulose, it possesses superior mechanical properties and a high degree of crystallinity.[6] Microbial cellulose, produced by bacteria has abundant importance in the field of life sciences, due to its unique structural properties in particular strength, foldability, and increased capacity to retain water, rather than plant cellulose.[7] Bacterial cellulose is currently being used in various commercial applications, including textile, cosmetic, and food goods, as well as for medical purposes, due to advances in its potential to be synthesized and characterized.[7] Bacterial cellulose's hydrophilic nature and indigenous dimensions idealize biocompatible nanocomposites with controlled mechanical properties.[7,6] Various types of Gram-negative bacteria, including *Acetobacter* sp*, Azotobacter* sp*, Rhizobium* sp*, Pseudomonas* sp*, and Salmonella* sp, have been found to produce cellulose.[8]

Isolation of bacterial cellulose-producing bacteria from agricultural wastes such as food, fruit, wheat straw, and cotton-based waste has been demonstrated previously. The advantage of utilizing agricultural wastes as a source and substrate provides cheap production costs and a clean environment.[9] *Acetobacter* species are commonly found in flowers, fruits, palm wine, vinegar, kefir, and fermented foods. In addition, they have been known to cause infections in grape wine, sake, tequila, cocoa wine, cider, beer, and fermented meat.[10] Acetic acid-producing bacteria are present naturally in spoiled fruits since they are considered an excellent medium for acetic bacteria due to the partial fermentation of substrate into alcohol.[10,11] The family Acetobacteraceae, acetic acid-producing bacteria, plays a decisive role in the synthesis of cellulose and acetic acid. Cellulose produced by the *Acetobacter* strain of microbes can be used as a dietary supplement as well as for manufacturing high-performance speaker diaphragms, medical and cosmetic pads, paint thickeners, and artificial skin.[12,13]

The present research investigates and targets isolation, identification, characterization, and optimization studies of the novel potential cellulose producer, rather than *Acetobacter xylinum*, that has been utilized as a prototype organism for basic and applied cellulose studies in numerous previous research works. The cellulose characterization and cytotoxic study were studied to employ it in an environment-friendly biopolymer production. Cellulose nanostructures have been utilized as reinforcing agents and matrices for various materials, such as films used in food packaging applications. Polysaccharide imparts crispness, compactness, hardness, thickening quality, adhesiveness, and gel-forming ability to a variety of films.[14] Bacterial Cellulose must be disintegrated to be employed in a powder (or solution) form for subsequent formulations, as for most food applications whilst bacteria produce it as a membrane.[15] Bacterial cellulose (BC) is particularly beneficial in these situations due to its unique characteristics. The edible biodegradable films serve as mass transfer barriers for moisture, oxygen, carbon dioxide, lipids, flavours, and odours between food products and the atmosphere, helping provide physical protection to food. These polymers have significant potential as their properties can easily be altered and the desirable features can be imparted at the same time with a wide range of bio disposal options such as soil burial method or microbial degradation method or

compost degradation method.[16] The key advantage of bacterial cellulose is its simplicity in top-to-down processing into microfibrils, nanofibrils, and nanocrystals.[17] Therefore, cellulose shall be employed for the production of biodegradable polymers for sustainable packaging as an environmental aid to reduce single-use plastic pollution.

MATERIALS AND METHODS

Sample collection

Cellulose-producing bacteria isolated from citrus fruit peel of orange (A1) and mosambi (A2), collected and peeled off. The aseptically collected peel samples were washed in sterile distilled water and subsequently crushed.

Enrichment of peel and isolation of cellulose-producing strains

To enrich the peel samples, 5 grams of samples were immersed in a solution containing 3% acetic acid and 4% ethanol. The mixture was then incubated at a temperature of 37°C for a period of three days.[8]

Enriched samples, were inoculated in 9 mL of saline (0.9%) and serially diluted up to 10^6 dilutions and were spread plated in the standard Hestrin-Schramm plating medium and incubated at 30°C for 48 hours. Loopful of each isolate inoculated in yeast peptone mannitol (YPM) broth and incubated statically at 30°C for 7 days. Pure culture of isolates obtained by subsequent streaking on Hestrin-Schramm agar plates.[8]

Screening of cellulose-producing strains

Two screening mediums of Hestrin-Schramm broth medium with antifungal agent cycloheximide and HS medium with a pinch of citric acid, urea inoculated with isolates from HS plating medium, and incubated at 30°C for 3 days.[18,8]

Characterisation of cellulose-producing strains

Various methods, including gram staining, colony morphology analysis, and biochemical characteristics assessment, were performed and compared according to Bergys manual of bacteriology to identify bacterial strains. Carbohydrate fermentation testing and plating in GEY and Carr medium were also conducted.[8-20]

Sequencing of cellulose-producing bacteria

The two most productive cellulose-producing isolates were chosen and completely identified with the 16S rRNA sequences analysis.[21]

Analysis of 16S rRNA Gene sequence

Cellular DNA isolation was performed and amplified 16S rDNA was done with the universal 16S primers.[22]

Phylogenetic relationships

The BLAST program[23] of The National Center for Biotechnology Information was used to compare the sequence of the isolates A1 and A2 with known 16S rDNA sequences. To determine the phylogenetic relationships, the MEGA (Molecular Evolutionary Genetics Analysis) program with the NJ (neighbor-joining) method was used. The gene sequence was submitted to secure the accession number.

Optimization of cellulose-producing strains

The impact of different medium components on the production of microbial cellulose has been studied with the two highly effective strains. Standard graphs are plotted to provide a visual representation of the findings. Different physiological and nutritional parameters, such as pH, temperature, carbon, nitrogen sources, and inoculum size examined to maximize cellulose production by the isolates in the static growth phase.[24-26]

Production of cellulose in an optimised hs medium

In an optimized Hestrin-Schramm media (D-glucose 2.0 g, yeast extract 0.5 g, peptone 0.5 g, disodium phosphate 0.27 g, and citric acid 0.15 g), 0.6% and 0.8% inoculum of each strain were inoculated and incubated at 30°C for 15 days and maintained at pH 7.[25,26]

Extraction and purification of cellulose

The bacterial cellulose was recovered (modified). Bacterial cellulose that has accumulated on the production medium's surface was scraped

off. The fermented broth centrifuged for 10 minutes at 10,000 rpm to remove cells, other medium ingredients, and the cells precipitated with cellulose. The cellulose pellet was treated with 0.1 N NaOH for 30 minutes at 90°C, then washed in distilled water to reduce pH of 5 until the wash water became neutral pH. It was then oven-dried at 65°C for 8 hours to remove any remaining moisture. The bacterial cellulose concentration is calculated on a dry weight basis according to the following formula.[27,21]

$$\text{Yield \%} = \frac{\text{dry cellulose production(g/l)}}{\text{original sugar(g/l)}} \text{x100}$$

Media constituent analysis

Media constituent analysis was performed to determine constituents utilized by the bacterial strains in the medium to produce bacterial cellulose.[28]

Lipid test

To 1 ml of fermented broth, 0.5 gm of potassium bi-sulphate was added and heated on a direct flame for 3 minutes. The odour of the mixture was checked for devoid of odour or the presence of a pungent fruity smell indicating the presence or absence of lipids in the medium.

Carbohydrate test

To 1.8 ml of fermented broth, 10 drops of freshly prepared benedicts reagent was added and kept in the water bath at 45°C for 5-10 minutes. The colour change of the mixture was observed before and after heating, indicating the absence or presence of carbohydrates in the medium.

Protein estimation

The protein estimation was determined by the biuret method. The standard solution of BSA and biuret solution was prepared. Distilled water was used to make up a total volume of 1 ml for concentrations of BSA at 0, 0.2, 0.4, 0.6, 0.8, and 1 ml. The fermented broth of 1 ml was taken as a sample. To each concentration, 3 ml of biuret reagent was added and incubated at 37°C for 20 minutes. The absorbance of the solution was recorded at 540 nm using a UV-visible spectrophotometer. A standard graph was plotted.

Characterisation of bacterial cellulose
Scanning Electron Microscope (SEM)

The fine details of bacterial cellulose's structure were examined using a scanning electron microscope (Tescan Vega III Easyprobe, Brno, Czech Republic). The dried cellulose samples were coated with gold, examined at an accelerated voltage of 15 kV and 10 kV with a magnification of 5000 k, and photographed.[8,29]

Fourier Transform Infrared Spectroscopy (FT-IR)

The functional groups of harvested bacterial cellulose and commercial cellulose were analyzed by FT-IR. FT-IR analysis of cellulose was determined after extraction, washed with Milli Q water, treated with 0.1 N NaOH, and dried. The dried samples were recorded using Shimadzu FTIR spectrophotometer with 400 to 4000 cm^{-1} wavelength. The spectra were recorded with a resolution of 2 cm^{-1} and accumulated over 32 scans.[1,30,29]

Cytotoxicity study of cellulose

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) rapid colorimetric assay was performed to estimate viable cell number. The data was analyzed by plotting cell number versus absorbance (Table 1).[31,32]

The A2 strain cellulose sample was sterilized to ensure sterility under UV light for 2 hours and further, the cellulose was dissolved in 2 ml of DMEM- high glucose media. The test agents were added in suitable concentrations, and the plates were then incubated for 24 hours at 37°C in an atmosphere containing 5% CO_2. After incubation, spent media was removed and MTT reagent was added, incubated for 3 hours. Followed by removal of the MTT reagent, 100µl of solubilization solution (DMSO) was added. Crystal formation by MTT was avoided by gentle stirring and Occasional pipetting up and down to completely dissolve the MTT. To calculate cell viability, the reference wavelength was measured using a spectrophotometer at 570 nm. The percentage of cell viability is then determined using the formula below:

Percentage of cell viability = (Mean abs of treated cells/Mean abs of Untreated cells) x 100

RESULTS AND DISCUSSION

Enrichment of peel, isolation, and screening of cellulose-producing bacteria

Among five bacterial strains, two strains produced evident white pellicle formation in broth and clear halos around the wells in the GEY plating medium. Figure 1 shows the culture plate of colonies fluoresce when observed under UV light. Figure 2 shows clear halos around the wells loaded with bacterial culture. The presence of fluorescent colonies and clear halos confirmed the isolation of cellulose-producing bacteria.

The technique of enrichment culture is useful in promoting the growth of acetic acid bacteria found in fruits. However, since only one enrichment medium was used in the study, there is a possibility that some types of acetic acid bacteria may not have been retrieved.[25] According to Sharafi et al.,[33] different types of flowers and fruits were good sources of acetic acid bacteria.

The bacteria's cell wall has pores through which glucose subunits are extruded to form the cellulose microfibril. The pellicle floats on the medium's surface, providing ample oxygen for the bacteria's growth, multiplication, and further cellulose synthesis.[34,35] The formation of a clear zone around the bacterial colony is caused by the absence of calcium carbonate.[36,25] As the colony grows, it produces acetic acid that reacts with $CaCO_3$, causing it to disappear and create a clear zone. This reaction produces calcium acetate, which is water soluble. Under UV light observation, the cellulose-producing bacterial colony fluoresces as it is bound to 5b-D glucans through a definable and reversible process in the screening medium containing urea and citric acid, whereas Rangasamy et al.[8] used calcofluor white as a fluorescent brightener dye. There were no contradictory results observed.

Table 1. Cell line study of cellulose produced by A2

No.	Test Compound	Cell lines	Concen. treated to cells
1.	Untreated	3T3-L1	Not treated
2.	Blank	-	Media without cells
3.	Std control	3T3-L1	4 µM/ml
4.	AB2	3T3-L1	5 (12.5, 25, 50, 100, 200 µg/ml)

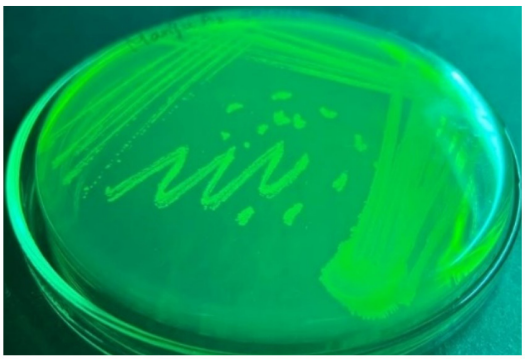

Figure 1. Culture plate of fluorescent colonies under UV light

Characterisation of cellulose-producing strains

Table 2 shows the biochemical characteristics of isolates A1 and A2, and Table 3 represents the results for cultural characteristics. They were Gram-negative, rod-shaped, catalase-positive, and oxidase-negative organisms, which are presumptive characteristics of acetic acid bacteria according to Bergey's manual.[37] The isolates were able to utilize glucose, sucrose, and mannitol as carbon sources with acid production whereas no gas production was observed in any of the carbon sources by both strains. Neither acid production nor gas production was observed in lactose as a carbon source. The strains showed positive results for indole, citrate, and urease; the triple sugar ion test showed acid production in slant and butt with gas production, and no hydrogen sulphide production was seen. Both strains showed a partial reduction of nitrate. The morphological, cultural, and biochemical

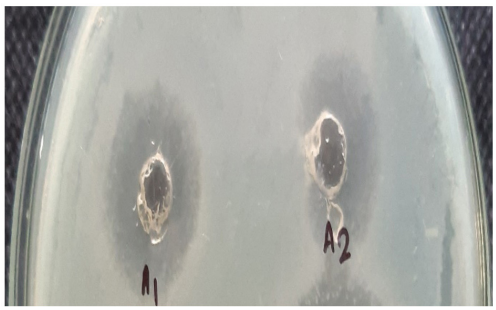

Figure 2. Clear halos around well loaded with bacterial culture A1 and A2 in GEY media

characters of the isolates show a significant profile that corresponds to the genus *Acetobacter* sp.

Two isolates showed media change of green colour to yellow, reversed back to green from yellow after prolonged incubation as characteristic of *Acetobacter* sp. (Figure 3), whereas incapability of media change by three strains considered to be *Gluconobacter* sp.

The two major acetic acid-producing genera were primarily selected based on their gram reaction, microscopic observation, catalase, and oxidase reactions.[38] *Acetobacter* strains consist of rod-shaped individual cells that occur as singles, pairs, or short and long chains. Gram-negative characteristics are present in young cells, while gram-variable characteristics are evident in old cells.[39] *Acetobacter* strains can be distinguished from *Gluconobacter* strains using the standard method outlined by Carr.[19] Carr medium consists of ethanol and bromocresol green as carbon source and pH indicator. *Acetobacter* strains were able to oxidize ethanol to acetic acid thus turning medium from green to yellow. However, acetic acid is over-oxidized to CO_2 and H_2O through the tricarboxylic acid cycle in neutral and acidic conditions with the reversion of green colour in the medium after an extended incubation period. This characteristic of *Acetobacter* sp is used to distinguish between members of the genus *Acetobacter* and *Gluconobacter.*[10] *Gluconobacter* is only capable of oxidizing ethanol into acetic acid. It cannot over-oxidize due to the non-functional α-ketoglutarate dehydrogenase and succinate dehydrogenase in its tricarboxylic acid cycle. In comparison,[39] contrast results have been observed with indole and urease tests. The carbohydrate utilization test results compared with the Arifuzzaman et al.,[25] had no contradiction.

Table 2. The biochemical characteristics of A1 and A2

TEST	A1		A2	
Indole	++		+	
Citrate	+++		+++	
Nitrate reduction	++		++	
Urease	+		+	
Carbohydrate fermentation	AC	Gas	AC	Gas
Glucose	+++	-	+++	-
Lactose	-	-	-	-
Sucrose	+	-	+	-
Mannitol	+	-	-	-
TSI	A/A		A/A	
Oxidase	-		-	
Catalase	-		+++	

+++ = strongly positive, ++ = moderately positive, += weakly positive, -= negative, A/A= acid slant, acid butt with gas

Table 3. The cultural characteristics of A1 and A2

Characteristic	A1	A2
Configuration	Round	Round
Elevation	Flat	Raised
Margin	Entire	Entire glistening
Surface	Smooth	Smooth
Colour	Yellow	Yellow
Opacity	Opaque	Opaque

Detection of cellulose

Two strains formed cellulose pellicles at the air-liquid interphase Figure 4. The pellicle remained intact after being alkali treated at 90°C with 0.1 N sodium hydroxide and rinsed with distilled water. Hence harvested cellulose pellet is considered pure cellulose.[8]

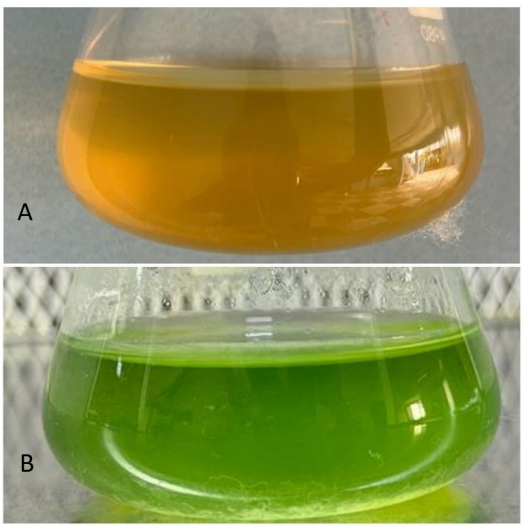

Figure 3. Colour change of isolate in Carr broth medium. A- during incubation period, B- reversion of green colour after 15 days of incubation

Sequencing of bacteria

Identification of bacteria solely based on phenotypic characteristics, such as cultural and biochemical traits, is not entirely reliable. Therefore, molecular technique 16S rRNA is most used to identify bacterial classification. After exploiting the Basic Local Alignment Search Tool (BLASTN 2.2.25) software to compare the nucleotide sequences of 16S rRNA genes from the most efficient isolates with sequences available from Gen Bank, it was found that the bacterial isolates belong to the genus *Acetobacter* sp. The bacterium was found to be similar to *Acetobacter lovaniensis* 16srRNA, partial sequence, AB 308060. The next closest homolog was found to be *Acetobacter lovaniensis* 16srRNA, partial sequence, JCM 17121. The isolate (A1) was identified to be *Acetobacter lovaniensis* 16srRNA, partial sequence with accession number OK 384569 as shown in Figure 5.

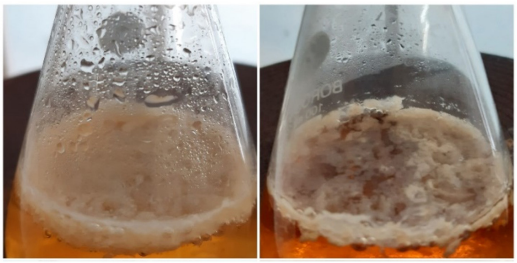

Figure 4. Pellicle formation by A1 and A2

The bacterium (A2) was found to be similar to *Acetobacter fabarum* 16s rRNA, partial sequence, MH 242619. The next closest homolog was found to be *Acetobacter fabarum* 16s rRNA, partial sequence, MT 611597. The isolate (A2) was identified to be *Acetobacter fabarum* 16s rRNA, the partial sequence with accession number OK 384568 as shown in Figure 6.

Optimisation of the medium

The fermentation medium contains nutrients, such as carbon, nitrogen, macro, and micronutrients, that are necessary for the growth of microorganisms. Slight variations in these components can have a direct or indirect impact on cell growth and product formation. It has been observed that the production of exopolysaccharides is more efficient when bacteria are provided with ample carbon sources and limited nitrogen sources. The results obtained

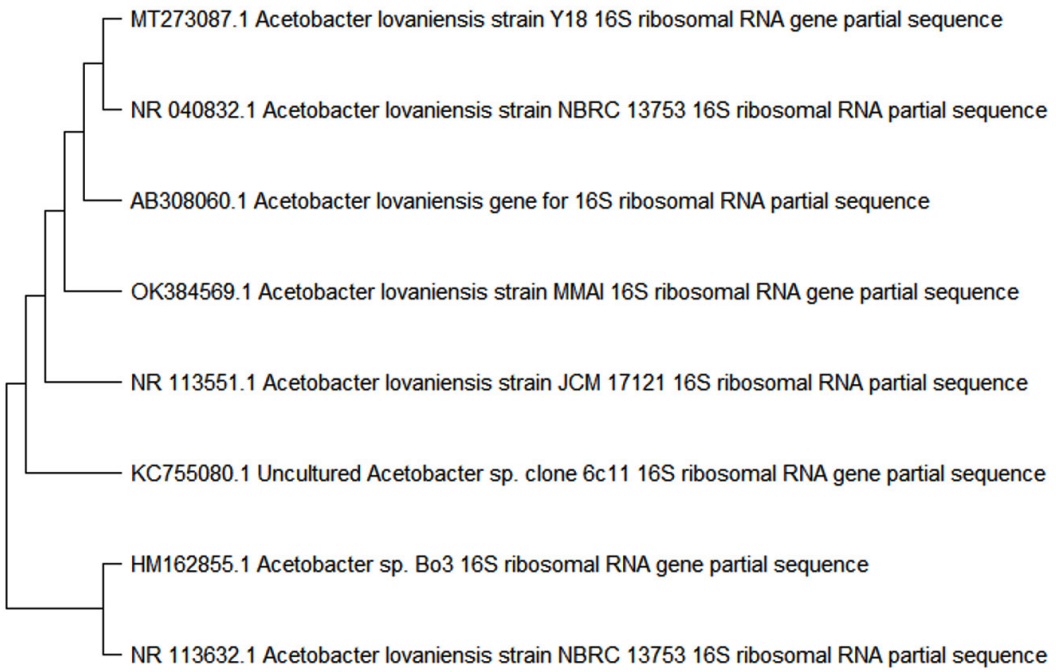

Figure 5. Phylogenetic tree relatedness to A1

prove that the optimization of cultural conditions for cellulose production is as significant as that of an organism.

Effect of inoculum size on cellulose production

The results for inoculum size of strains ranging from 2% to 12% (v/v) were examined and a graph was plotted in Figure 7. Inoculum sizes of 6% and 8% from A1 and A2 showed the highest cellulose production, corresponding to 0.7 g/l and 0.69 g/l. Inoculum sizes of 2% and 12% illustrated the lowest yield of cellulose with both strains.

Cellulose production is greatly affected by the volume of inoculum, as stated in the study.[40,8] However, in this particular study, all the parameters of optimization for increased cellulose

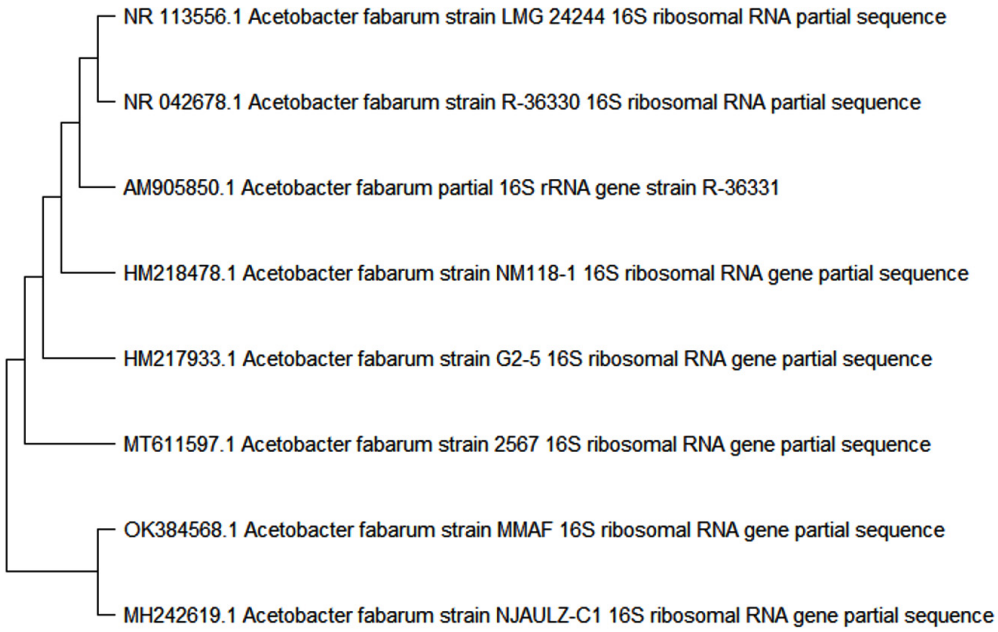

Figure 6. Phylogenetic tree relatedness to A2

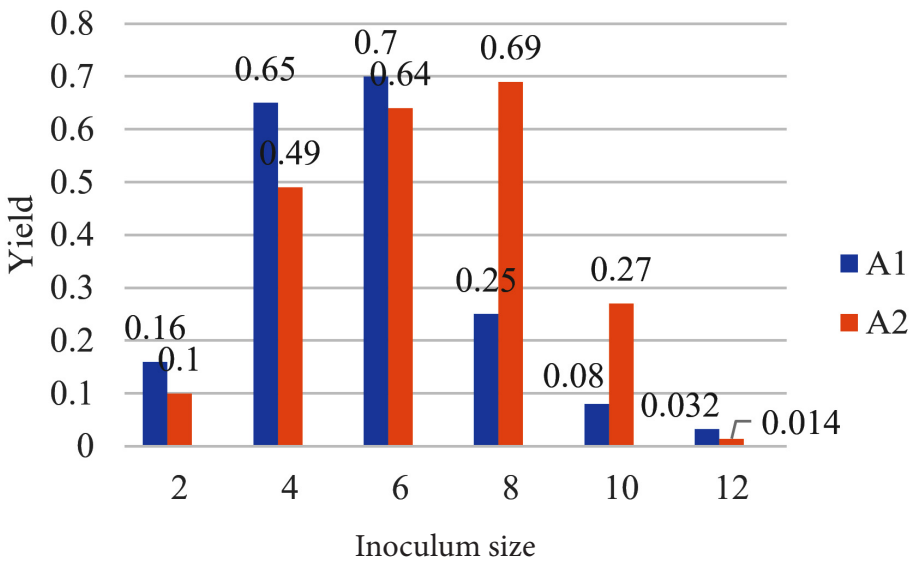

Figure 7. The effect of inoculum size on cellulose

production could not be measured using the OD method due to the transparent nature of the media and pellicle formation at the air-liquid interphase that was observed within 48 hours of incubation. Extraction with alkali treatment has been carried out to determine the yield of cellulose. According to numerous researchers, the number of cells present in the aerobic zone responsible for cellulose production is more important than the total count of cells for optimal cellulose production. Therefore, the results obtained lead to the conclusion of an optimum inoculum load of 6% with A1 and 8% with A2.

Effect of pH on cellulose production

Figure 8 represents the yield of the strains cultured in a medium ranging from pH 5 to 8 and the yield was quantified with equation 1. In comparison 5,6,8, and pH 7 showed the highest production of cellulose. Thus, pH 7 is ideal for the formation of cellulose, yielding a maximum of 0.726 g from strain A1, and 0.864 g from strain

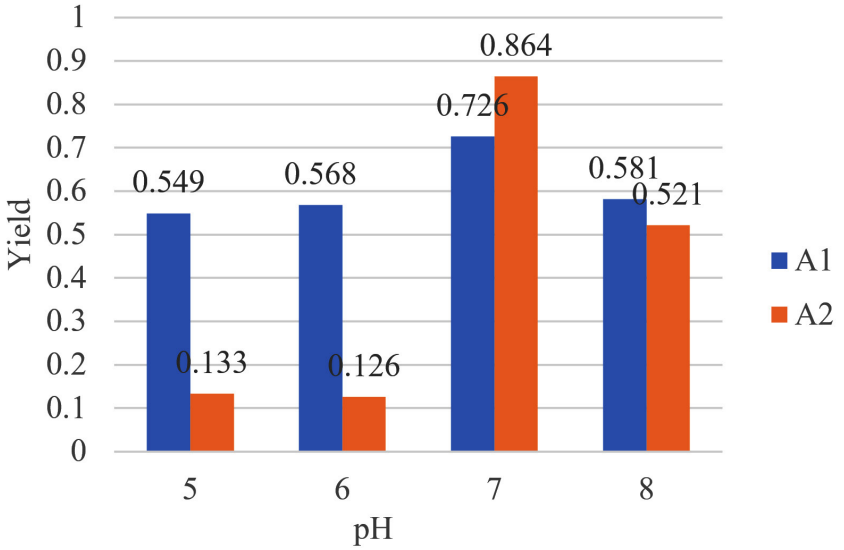

Figure 8. The effect of pH on cellulose production

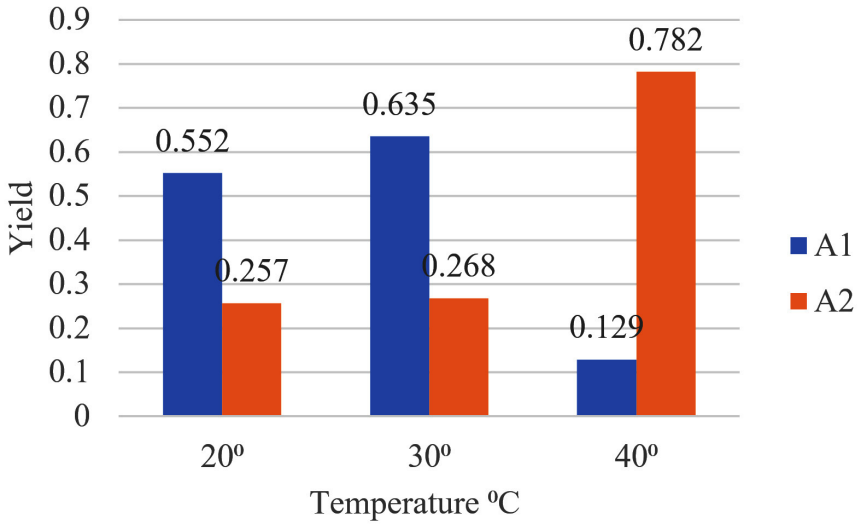

Figure 9. The effect of temperature on cellulose production

A2 in comparison to other pH values. The pH 7 showed increased production of cellulose with both isolates followed by pH 8. The isolate A2 shows very less cellulose production with pH of 5 and 6. The isolate A1 shows the least cellulose production with pH of 5.

According to Rangaswamy, [8] Maintaining the correct pH level is vital for *Acetobacter* to produce high-quality products. If the culture's pH drops below 4 due to the build-up of gluconate, cellulose synthesis reduces. As soon as the glucose concentration in the media is oxidized, the bacteria start metabolizing gluconate, resulting in a gradual increase in culture pH. When the pH for cellulose synthesis reaches above 4, cell division resumes. Therefore, the present study concludes that the ideal pH for cellulose production is 7 and the results obtained coincide with the study of Chawla et al.[24] and Arifuzzaman et al.[25]

Effect of temperature on cellulose production

The results on cellulose production temperature range from 20°C to 40°C were examined and plotted in Figure 9. The A1 organism produced the highest cellulose at a temperature of 30°C, yielding 0.635 g/l, while the A2 organism

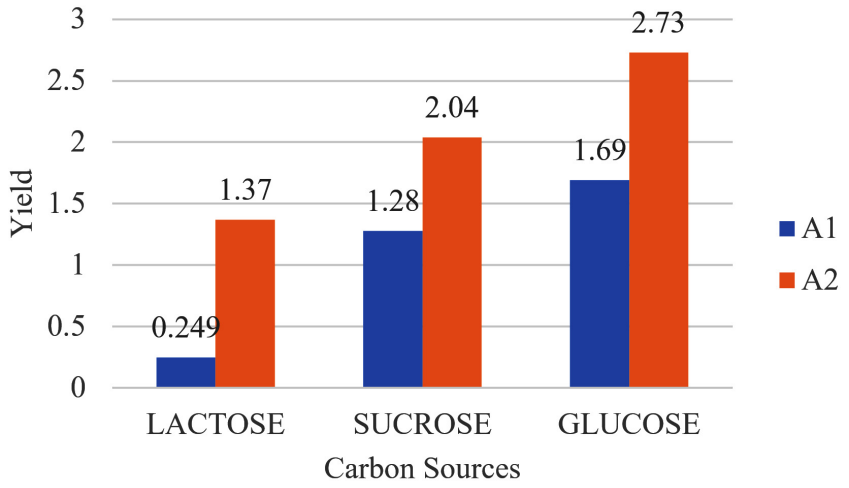

Figure 10. The effect of carbon sources on cellulose production

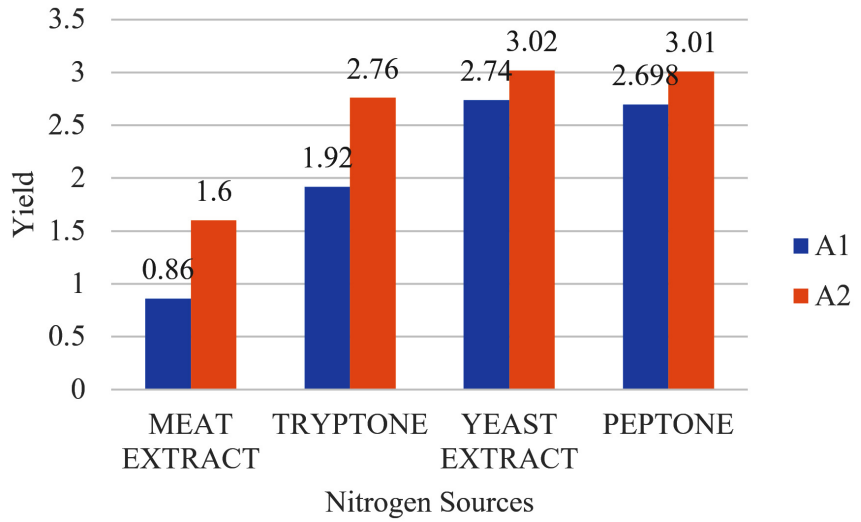

Figure 11. The effect of nitrogen sources on cellulose production

produced its most cellulose at a temperature of 40°C, yielding 0.782 g/l. At the temperature of 20°C A2 isolate shows the least cellulose production with a yield of 0.257 g/l. The cellulose production was the least and not seen by both strains in the range of 45°C. The Least cellulose production of A1 was observed at 40°C with a yield of 0.129 g/l, whereas A2 showed the least production at 20°C with a yield of 0.552 g/l.

The optimum temperature for acetic acid bacteria growth ranges from 25°C to 35°C.[25] The incubation temperature was maintained at 30°C and observed that *Acetobacter* strains were able to grow at 25°C and not below 20°C. Acetic acid bacteria are mesophilic in nature with an optimum growth temperature of 30°C.[41] However, some are able to grow at 37°C and 40°C which are thermotolerant strains.[6] The present research concluded that cellulose production is best at temperatures between 30°C and 40°C and no contradictory results were observed.

Effect of carbon sources on cellulose production

Carbon supplements like sucrose, lactose, and glucose supplied at 2% (w/v) in a typical Hestrin-Schramm medium to evaluate the impact of carbon sources on the yield of cellulose has been illustrated in Figure 10. The strains used every carbon source, with lactose accounting for the least amount with 0.24 g/l with strain A1 and 1.37 g/l with strain A2. The highest levels of cellulose production were observed in glucose, with a yield of 1.69 g/l in strain A1 and 2.73 g/l in strain A2, followed by sucrose with a yield of 1.28 g/l from A1 and 2.04 g/l from A2.

Cell development and the formation of cellulose rely solely on carbon. The most efficacious cellulose production is obtained with glucose as the primary carbon source.[42] Cellulose synthesis is a series of multi-step chemical reactions, with glucose catalyzed by enzymes.[35] The glucose subunits form the microfibril of cellulose and extrude through pores of the bacterial cell wall.[34] Glucose is the main carbon source for the extracellular formation of bacterial cellulose by *Gluconabacter xylinus*.[21] Glucose, mannitol, and sucrose were found to be the optimal carbon sources for cellulose production by *A. xylinum* NCIM 25526.[42] Unlike other carbon sources, glucose can be directly used for cellulose synthesis.[43] However, glucose metabolism leads to an increase in gluconate and a decrease in pH. Studies show that the effectiveness of cellulose production by *Acetobacter* is determined by the availability of carbon sources and the build-up of metabolic by-products that create unfavourable growth conditions.[24] The present study concludes that the ideal carbon source for cellulose production is glucose. No contradictory results were observed with above discussed research work.

Effect of nitrogen sources on cellulose production

The nitrogen sources peptone, tryptone, yeast extract, and meat extract were supplied at 0.5% (w/v) in a standard HS medium to evaluate

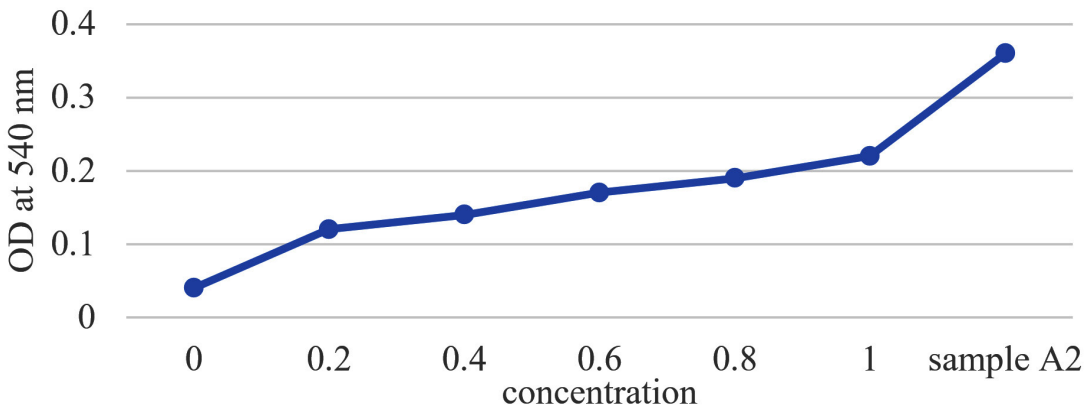

Figure 12. The protein estimation of sample A2 with biuret method

the impact of nitrogen source on the yield of cellulose, and the results are illustrated in Figure 11. The interpretation of the results shows that strain A1 with peptone produced 2.69 g/l whereas strain A2 produced 3.01 g/l of cellulose. Yeast extract produced the highest cellulose production

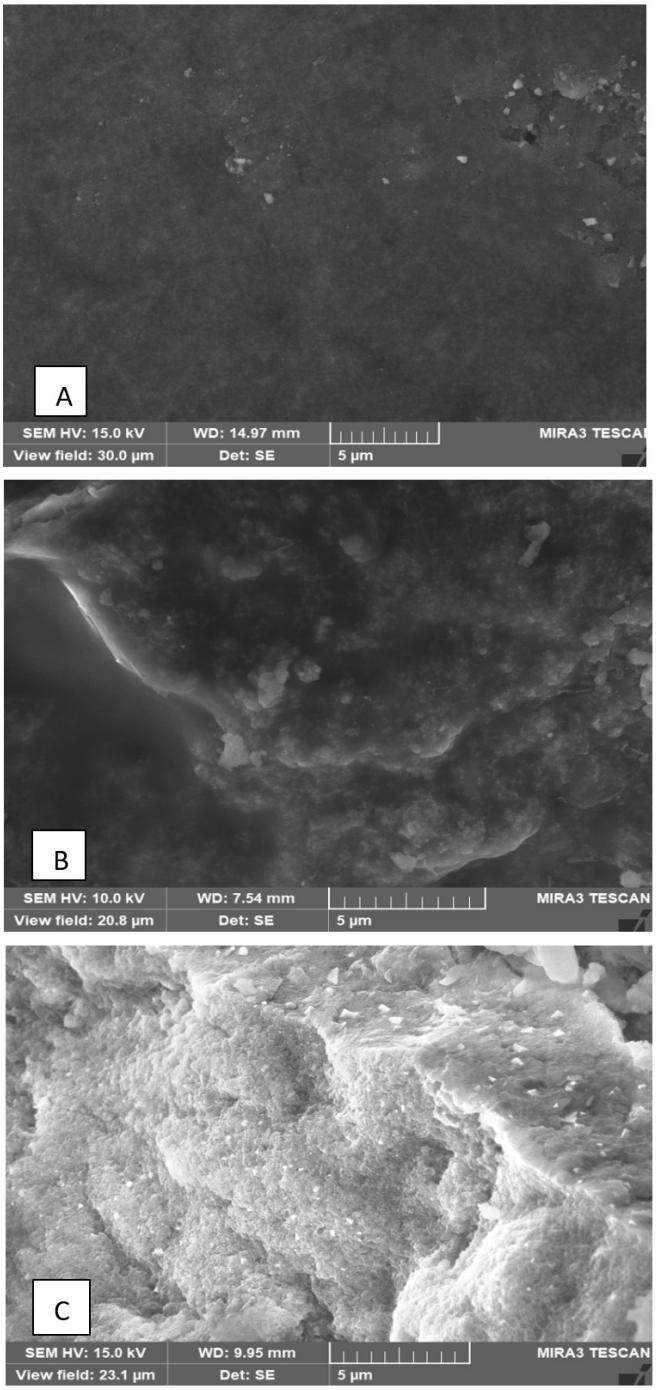

Figure 13. The SEM image of cellulose samples. A- commercial cellulose, B- cellulose from A1, C- cellulose from A2

rate of 2.74 g/l with strain A1 and 3.02 g/l with strain A2. Tryptone produced 1.9 g/l and 2.76 g/l with strains A1 and A2, respectively. The least cellulose production was observed with meat extract of yield 0.86 g/l from A1 and 1.6 g/l from A2.

The nitrogen source is essential to produce cellulose and cell metabolism. A specific complex

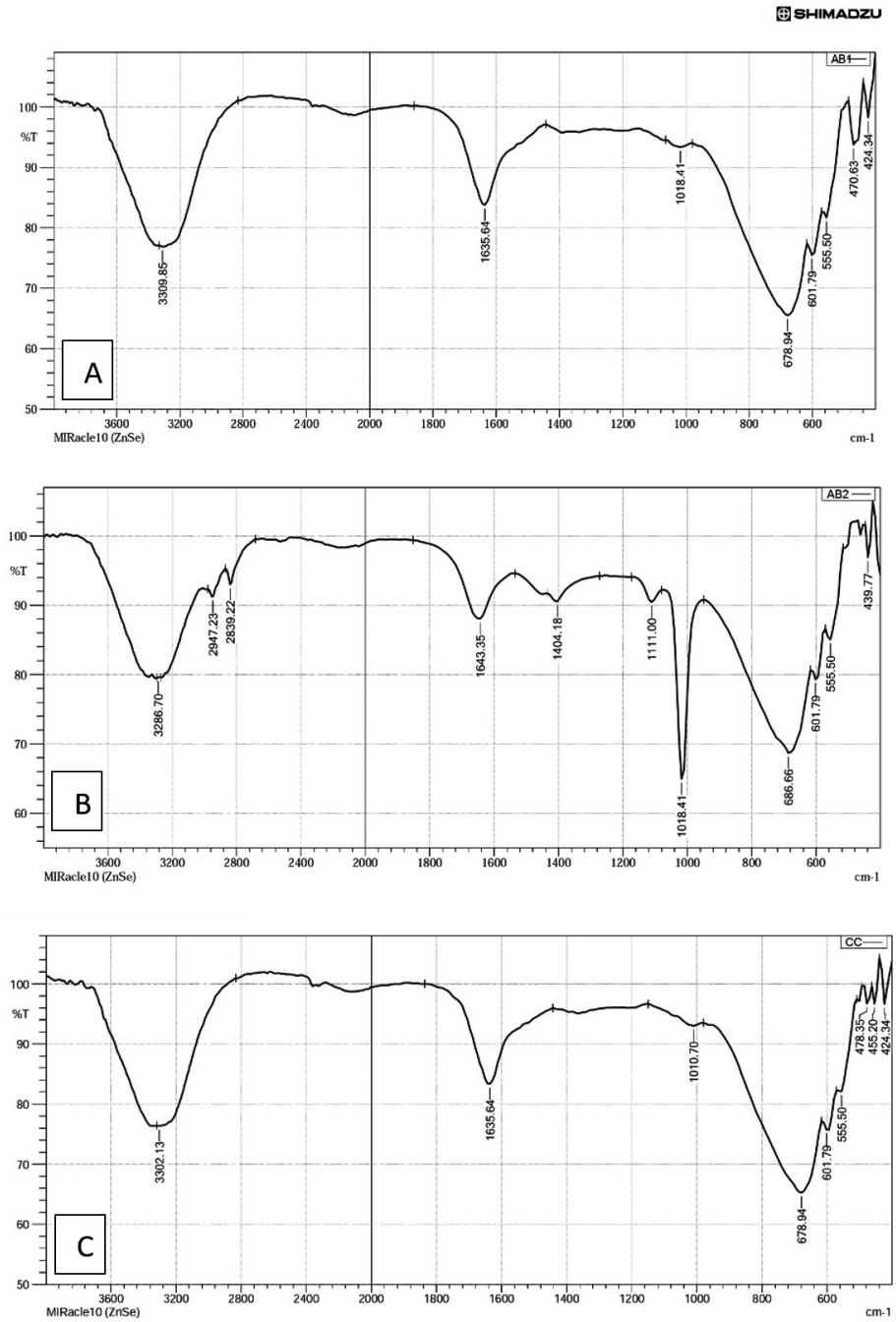

Figure 14. FT-IR peaks of cellulose samples. A- cellulose of A1, B- cellulose of A2, C- commercial cellulose

nitrogen source is required for cellulose-producing strain. Incorporation of inorganic nitrogen sources into the medium shows a low level of cellulose production. No growth and cellulose production was observed,[44] when inorganic nitrogen sources were supplemented to isolate *G. xylinus*. Among various nitrogen sources to assess the effect of cellulose production,[45] the best nitrogen source for the production of cellulose by *Acetobacter* sp is yeast extract. Many researchers have reported yeast extract to support maximum bacterial cellulose production.[21] The study has led to the conclusion that yeast extract and peptone are the preferred nitrogen sources tested in the study of increased cellulose synthesis. No contradictory results were observed.

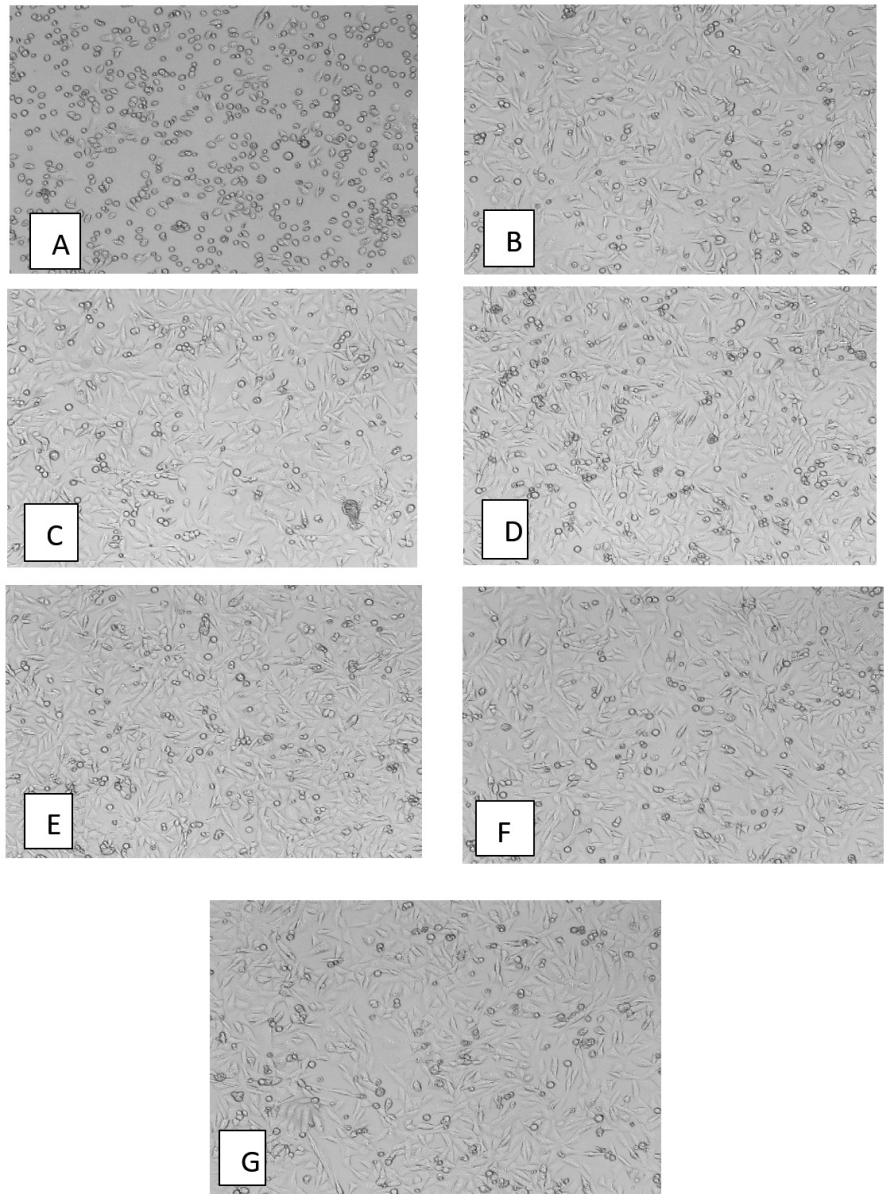

Figure 15. Represents microscopic observation of cell viability with test compound cellulose A2. A- untreated, B- standard control, C- 12.5 µg/ml, D- 25 µg/ml, E- 50 µg/ml, F- 100 µg/ml, G- 200 µg/ml

Table 4. Band Assignment of FT-IR Spectra of Cellulose Samples

Wave No. (cm^{-1})	Functional Group	Intensity
3309	Alcohol O-H stretching	Strong
3302	Alcohol O-H stretching	Strong
3286	Alcohol O-H stretching	Strong
2839	C-H aldehydic	Variable
2947	C-H stretching	Weak
1635	Amide C=O	Strong
1643	Amide C=O	Strong
1404	CH bending	Weak
1111	C- OH stretching	Strong
1018	C-O-C stretching	Strong
1010	C-O-C stretching	Strong

Production and extraction of cellulose in an optimised hs medium

The modified HS medium inoculated with isolates shows quantified cellulose yield according to eq 1. The cellulose yield of the A1 isolate was 0.715 g/l, whereas the cellulose yield of the A2 isolate was 0.856 g/l.

Earlier studies of bacterial cellulose production used *Acetobacter xylinum* as the predominant organism. Therefore, we have made an effort to explore other species from the genera *Acetobacter* for cellulose production, *Acetobacter lovaniensis* and *Acetobacter fabarum* were identified and utilized. Previous studies have shown that cellulose synthesis is part of primary metabolism, which has been observed in several bacterial species, including *Acetobacter xylinum, Rhizobium leguminosarum, Klebsiella pneumoniae, Sarcina ventricle, Agrobacterium tumefaciens, Salmonella typhimurium, Escherichia coli, Enterobacter, and cyanobacteria.*[8] Schramm and Hestrin have discovered the ideal conditions to produce cellulose, as outlined in the study.[42] Previous research has shown that *Acetobacter xylinum*, a Gram-negative, obligate aerobic bacterium, is a commonly studied archetype for cellulose synthesis.[24] Many of these studies have found that the effectiveness of cellulose production in *Acetobacter* is primarily determined by the availability of carbon sources and the accumulation of metabolic by-products that lead to unfavourable growth conditions.[24] A mechanical separation technique and an alkali treatment process were developed to extract bacterial cellulose while eliminating bacterial cells.[46] Extraction of cellulose was carried out with alkali treatment since cellulose was resistant to the treatment (remained undissolved) and accepted to be pure cellulose. Sodium hydroxide does not change the allomorphic structure of cellulose since low concentrations have been used.[8]

Table 5. The MTT assay cell viability against cellulose sample A2

Culture condition (µg/ml)	% cell viability
Untreated	100
Std control	66.02
12.5	97
25	93
50	89
100	80
200	73

Media constituent analysis

Lipid test

A pungent fruity odour was observed, indicating the presence of lipids in the medium. The test indicates that the organism did not utilize the lipid source for cellulose production.[28]

Carbohydrate test

No colour change was observed after heating. Therefore, indicating that the organism has utilized the carbohydrate source for cellulose production.

Protein estimation

The concentration of protein present in 1 ml of sample is 900 µg/ml. Therefore, indicating that the organism has not utilized protein source for cellulose production (Figure 12).

Characterisation of bacterial cellulose

Scanning Electron Microscope (SEM)

The crystalline structure and the external morphology of the compound cellulose pellicle samples were observed and analyzed in Figure 13. It can be observed that the ultrafine structure

of bacterial cellulose is crystalline in nature rather than commercial cellulose. Micro and nanofibrils of cellulose from A2 shall be evidently seen through SEM studies.

According to Umamaheshwari et al.,[29] SEM images from previous research findings showed the crystalline structure of bacterial cellulose. It has been observed that crystalline-natured cellulose was previously churned out by *Actinomycetes* sp*, Pseudomonas* sp*, Lactococcus lactis, and Gluconacetobacter* sp. Recent studies have found that glucose serves as a carbon source for bacteria to generate a crystalline type of cellulose in the following strains *Gluconacetobacter* sp*, Acetobacter xylinum* sub sp*. Sucrofermentans BPR2001, Achromobacter* sp*, Acetobacter aceti, Gluconacetobacter xylinus* strain ATCC53524.[8] It is crucial to comprehend the characteristics of materials, and their correlation to structure and chemical composition is significant. When examining the organization of cellulose, the sole dependable approach is to utilize a scanning electron microscope.[8] The SEM images of the ultrafine crystalline cellulose structure produced by A1 and A2 demonstrate that cellulose derived from *Acetobacter* sp has been proven to have high water-holding capacity. A previous researcher has made a statement about this property.[47] No contradictory results have been observed with the above discussed authors.

Fourier Transform Infrared Spectroscopy (FT-IR)

The significant functional groups of bacterial cellulose and commercial cellulose obtained have been discussed below and are similar to the typical FT-IR spectra of natural cellulose obtained from plants Figure 14. The band assignment of spectra is tabulated in Table 3 and 4. The cellulose crystalline structure can be observed at absorbance peaks of 3309 cm^{-1} in A1, 3286 cm^{-1} in A2, and 3302 cm^{-1} in commercial cellulose indicating cellulose's O–H stretch. C–H aldehydic stretching can be observed in sample A2 at the 2839 cm^{-1} region indicating the chemical composition of the main chain of bacterial cellulose. The peak at region 1404 cm^{-1} in A2 sample indicates the scissoring vibration of the -CH functional group. The peaks at 1635 cm^{-1} in A1 and 1643 cm^{-1} in A2 show the presence of the carbonyl amide group in bacterial cellulose. The peak at 2947 cm^{-1} in A2 is associated with the stretching vibrations of C-H groups, while the peak at 1643 cm^{-1} indicates the deformational vibrations of –OH groups that come from bound water. A series of bands from 1100cm^{-1} - 1000cm^{-1} from all the three samples was due to the stretching of C-O-C of sugar rings and C-O stretching vibrations of the primary (C6) and the secondary hydroxyl (C2, C3) groups. The asymmetric and symmetric stretching vibrations in region 1111 cm^{-1} from A2, 1018 cm^{-1} in A1 and A2, respectively, depict typical bacterial cellulose. The peak region between 850 cm^{-1} - 550 cm^{-1} of the samples indicates C-Cl stretching. The results obtained confirm three cellulose samples exhibited similar chemical binding.

The absorption bands are a fingerprint that confirms cellulose structure and slightly vary between different origins of cellulose.[48] The signature peaks of bacterial cellulose[29] have been observed, with no contradictory results to the present study. The cellulose's crystalline structure can be viewed at the 3340cm^{-1} absorbance peak, which indicates the O-H stretching of cellulose.[29] The intensity of the peak between 850-1150 cm^{-1} signifies that the bacterial cellulose's crystallinity with treatment at 90°C increased its crystalline content in comparison to treatment at 70°C.[1]

The FT-IR spectrum of bacterial cellulose samples shows no contradictory results from the reported spectra.[30,1,48,9,49]

Cytotoxicity study of cellulose

The cell line morphology was studied under a light microscope after incubation. The cells seemed to be affected very mildly by the contact of the test compound. Based on the MTT assay, it has been found that the test compound exhibits minute cytotoxic effects on the Mouse embryo fibroblast (3T3-L1) cells. The cell's shape and density did not differ noticeably from the control in any way. With an increase in concentration, the cell density decreased, especially when the concentration of the test compound was 50- 200 µg/ml. It showed that the compound had no apparent negative effects on the cell growth when the concentration of the test compound in the culture medium was from 12.5 µg/ml and 25 µg/ml. This is very consistent with the results from

the MTT assay. Therefore, it has been determined that the test compound is much less toxic with 97% viability of mouse embryo fibroblast cells. Additionally, it appears from visual observations that the examined nanofibril-based structures had less impact on the proliferation of the cells. The dependence on cell viability of fibroblast cells on cellulose sample concentration is represented in Table 5. When the concentration of cellulose was 12.5µg/ml the cell viability was 97% which is close to untreated cells. Figure 15 shows images of drug-treated test compounds exemplified under a microscope after 24 hours of incubation. The cellulose nanowhiskers[32] showed low toxicity with 0.1 and 0.2% in cell lines with viability of 96% and 78%. Further research is required to determine the variables that affect cellulose's cytotoxicity and the interactions of cellulose with cells or tissues. However, more research is needed to determine the molecular mechanism underlying its in vitro cell proliferation capabilities.

CONCLUSION

We have successfully isolated and conducted an in-depth study of two novel bacterial strains, namely *Acetobacter lovaniensis* A1 and *Acetobacter fabarum* A2, that demonstrate outstanding bio-cellulose production capabilities. The screening and characterization study of isolates and cellulose has been performed. FTIR and SEM analysis shows characteristic peaks and crystalline structure of the obtained bacterial cellulose. Additionally, we have thoroughly analyzed their cytotoxicity, allowing us to gain valuable insights into their potential applications. The cytotoxicity test results obtained show, that the novel isolate *Acetobacter fabarum* exhibits cellulose that is much less toxic in nature with 97% viability of cell line. Polysaccharide-based films act as a renewable source and are good in crispness, compactness, hardness, thickening quality, adhesiveness, and gel-forming ability to a variety of films and shall be employed to manufacture cellulose on a large industrial level with the outcome of a fruitful application to engender biodegradable food wrapper to reduce fritter away non-degradable disposable packaging.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This article does not contain any studies on human participants or animals performed by any of the authors.

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