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Evaluation of different carbon, nitrogen sources and industrial wastes for bacterial cellulose production

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Abstract

In this study we isolated an acetic acid bacterial strain from a home-made wine sample and investigated its cellulose production. The strain was identified using biochemical tests and 16S rRNA gene sequencing and named at the species level as *Acetobacter pasteurianus*. We investigated cellulose production by *Acetobacter pasteurianus* under the utilization of various carbon (glucose, sucrose, fructose, and ethanol) and nitrogen (yeast extract, casein hydrolysate, and ammonium sulphate) sources and industrial wastes (molasses, corn steep liquor, whey, and olive mill wastewater). The results showed that the best carbon and nitrogen sources for the production of cellulose were glucose and yeast extract. In addition, the best production occurred when molasses was used as the industrial waste. Bacterial and plant celluloses are reticulated and unbranched polymers of β-1,4-linked glucopyranose units. Therefore, the structural characteristics of cellulose were examined using Thin-layer chromatography (TLC), Fourier transform infrared spectroscopy (FTIR) and Carbon-13 nuclear magnetic resonance (C^{13}NMR) methods. The structural characteristics of bacterial cellulose were similar to those of plant cellulose. In addition, the porous and reticulated structure of bacterial cellulose was viewed using Scanning electron microscopy (SEM).

Keywords: *acetobacter pasteurianus*, cellulose production, SEM, FTIR, C^{13}NMR

1. Introduction

One of the most important materials in the industry is cellulose. In recent years, with advancements in biotechnology, biotechnologists tried find a way to produce cellulose without plants. They focused on cellulose producing bacteria. Researchs showed Acetobacter xylinum produces cellulose which is similar to higher plants [1, 2].

Cellulose is made of D-glucopiranose units linked with β-1, 4 bonds with non-water soluble, non-branched structure. Plant and bacterial cellulose are similar in chemical manner but their macromolecular structure and physical features are different [3]. Although plant cellulose has a structural role, bacterial cellulose acts as a protective coat. Bacterial cellulose fibrils are 100 times smaller than plant cellulose, high in polymerization, water absorbtion capacity, tensile strength and crystallinity [4]. Bacterial cellulose is accepted Generally Regarded As Safe (GRAS). This gives reason to use safely in foods [5]. Due to the not containing lignin and hemicellulose, bacterial cellulose is enviroment friendly and more advantageous for industrial use. Production yield of bacterial cellulose is 10000 kg/ha per year while cotton's cellulose production yield is 600 kg/ha per year [2]. Because of this, bacterial cellulose has a wide area of usage in paper, leather, food, cosmetics, textile and medicine [1]. Carbon and nitrogen source is important for cellulose production yield and to lower the costs, materials like molasses, sugar syrup, fruit juices, saccharose, whey, corn and potato wastes can be used [6, 7, 8].

Due to the statistics of Food and Agriculture Organisation (FAO) in 2011, paper and cardboard production in World was 390.7 million tons while Turkey's 2.847.326 tones. But, our country can not supply with cellulose need and imports it. This is a big load on country's economy [9]. To find a solution to this problem, in recent years, studies like this became important in Turkey.

The aim of study was to isolate Acetobacter sp. and investigate their cellulose production yield. For this purpose, Acetobacter pasteurianus (HBB6) strain was isolated from homemade wine sample as an acetic acid bacterium and identified with both classical and molecular methods. Cellulose production of isolated strain was researched in basal HS medium. Different carbon (glucose, sucrose, fructose, and ethanol), nitrogen (yeast extract, casein hydrolysate, and ammonium sulphate) and industrial wastes (molasses, corn steep liquor, whey, and olive mill wastewater) were tested for cellulose production yield. Structural properties, purity and chemical similarity with plant cellulose analysed with FT-IR, CP/ MAS 13C-NMR ve TLC methods. In addition that nanofibril structure of cellulose was screened with SEM.

2. Material and Methods

2.1 Isolation of microorganism

Ten mililiter of wine samples were taken under aseptic conditions and added on 90 ml of HS Broth (20 g/l glucose, 5 g/l yeast extract, 5 g/l polypeptone, 2.7 g/l Na2HPO4 and 1.15 g/l citric acid) [10]. 100 ppm cycloheximide was added in to the medium for inhibition of yeasts and fungi. Under static conditions, bacterium was incubated at 30oC for 7 days. After incubation, pellic formation was observed. Pellic was suspended in 0.85 % FTS (NaCl) solution under aseptic conditions and ensured that pellic was completely fragmentize. After complete fragmentization, it was diluated up to 10-3 with sterile saline solution. After diluation, from every diluate it was distributed homojenically to HS (1.5 % agar) and GYC agar (50 g/l glucose, 10 g/l yeast extract, 30 g/l CaCO3, 20 g/l agar) and incubated at 30oC for 7 days. At the end of the time period the little, white or beige, high or convex and compact mukoid colonies were selected. All isolates were preserved by freezing in glycerol at −20oC and monthly checked for strain stability [11, 12].

2.2 Identification of microorganism

Morphological and biochemical identification of isolates was performed using standard biochemical tests (catalase, oxidase, indole production, carbohydrate fermentation, nitrate reduction etc.) as outlined in Bergey's Manual of Systematic Bacteriology [13]. DNA isolation was carried out with classical phenolchloroform method according to Gonza´lez *et al*. [14]. 16 S rDNA universal primers were used (F: 5' AGA GTT TGA TCC TGG CTC AG 3', R: 5' ACG GCT ACC TTG TTACGA CTT 3) for PCR. PCR conditions were carried out in 10X Taq Buffer (Fermentas), 200 µM dNTP Mix (Fermentas), 7.5 mM MgCl2, 20 pmol of each primer, 2 µL DNA, 1 U Taq polymerase (Fermentas). Samples were incubated for 10 min at 94oC and 35 times at 92oC for 1 min, 48oC for 1 min and 72oC for 90 sec. The samples were incubated at 72oC for 15 min for final extension and kept at 4oC until tested (TECHNE TC 3000).

PCR products were sent to Macrogen Inc. (Seoul, South Korea) for sequencing. Sequences was compared with those present in the GenBank database using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

2.3 Production and purification of cellulose

Hestrin-Schramm medium (HS) was used as basic medium. The HS medium for growth and cellulose production comprised 20 g/l glucose, 5 g/l yeast extract, 5 g/l polypeptone, 2.7 g/l Na2HPO4 and 1.15 g/l citric acid [10]. The pH of the HS medium was adjusted to 6.0 with 1.0 M HCl.

For preculture, stock culture was inoculated into 100 ml of the basal medium in a 250 ml Erlenmeyer flask, and culture was incubated at 30oC for 48 h under static conditions. The culture was started by inoculating 1% of the culture supernatant from preculture. For static-flask fermentation, cultivations were performed at 30oC for 7 days under static conditions.

After cultivation, the culture medium was separated into supernatant and cellulose pellicle. The pellicle was treated with 4 % (w/v) NaOH solution at 80oC for 1 h to eliminate bacterial cells and medium components. Pellicle was rinsed with 6 % acetic acid until the pH of water became neutral. The purified cellulose was dried at 80oC and then weighed [8].

2.4 Effects of carbon, nitrogen sources and industrial wastes on cellulose production

To determine the effects of different carbon sources, we used glucose, sucrose, fructose and ethanol at 2 % concentration. Furthermore, the nitrogen sources casein hydrolysate and ammonium sulphate were used with each of the above carbon sources at a concentration of 0.5 % instead of yeast extract in the HS medium [6, 15]. In addition, molasses, corn steep liquor, whey and olive mill wastewater as industrial wastes were used for cellulose production [7, 8, 16].

2.5 Scanning electron microscopy of cellulose

For microscopic observations, cellulose was washed with 50 mM potassium phosphate buffer (KPB) (pH 6.5) and fixed with 2.0 % glutaraldehyde in the same buffer. Cellulose was dehydrated by washing with 50 to 100 % ascending ethanol series, and then lyophilised [17]. Then, the morphology of the cellulose was monitored by using a JEOL/JSM-6335F scanning electron microscope at TUBITAK, Turkey.

2.6 Determination of the total sugar amount in cellulose and sugar composition

Cellulose concentration was calculated from the dry weight of purified cellulose. Concentration was determined by the phenol/sulphuric acid method using glucose as a standard [18].

TLC (Alugram® Sil G/UV254 0.20 mm, 5x10 cm, Germany) was used to analyse formed products with trifluoroacetic acid (TFA) hydrolyses of cellulose.

Lyophilised cellulose samples (2 mg) were hydrolysed in an aqueous solution of TFA (2 M) for 2 h at 120oC in a screw-capped glass vials. Afterward, the resulting solution was evaporated at 40oC to remove the TFA [19]. The remaining portion was dissolved in 1 ml of distilled water. TLC of the hydrolysis products was performed according to Fontana *et al*. (1988) [20].

2.7 Fourier transformed infrared (FT-IR) spectroscopy

The surface properties of lyophilised cellulose was analysed using an FT-IR (VARIAN 800 FT-IR, Shimadzu Corp., Japan) spectrometer. FT-IR spectroscopy spectra were scanned in wave number range of 4000-550 cm-1. The powdered samples were mixed into KBr and analysed [21].

2.8 CP/MAS 13C NMR analysis

CP/MAS 13C NMR measurements were performed on lyophilised cellulose with a Bruker Biospin Ultrashield TM (300 MHz) spectrometer using the method of Yamamoto *et al*. [22].

3. Results and Discussion

3.1 Microorganism

Acetobacter pasteurianus HBB6 is a rod shaped, aerobic, Gram-negative, non-spore forming, catalase-positive bacterium (Fig. 1).

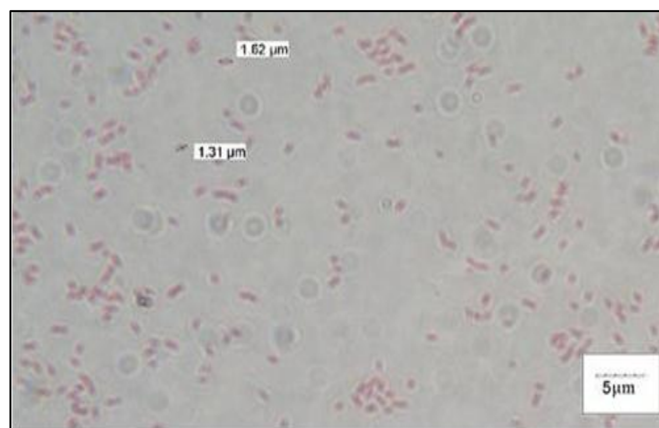

Fig 1: Image of the cell morphology of Acetobacter pasteurianus HBB6.

The isolate was inoculated on HS agar and CaCO3 agar plates and incubated at 30oC for 3 days (Fig. 2a, b). Then, small and beige colonies produced in plates were selected and stored at 4oC.

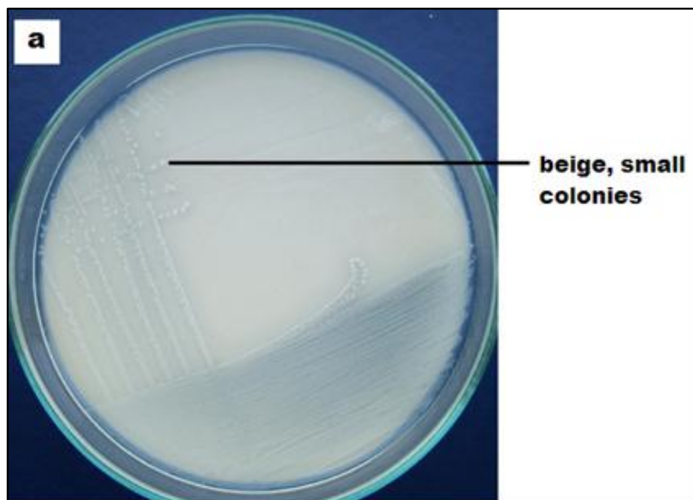

Fig 2a: Image of Acetobacter pasteurianus HBB6 colonies on HS-agar.

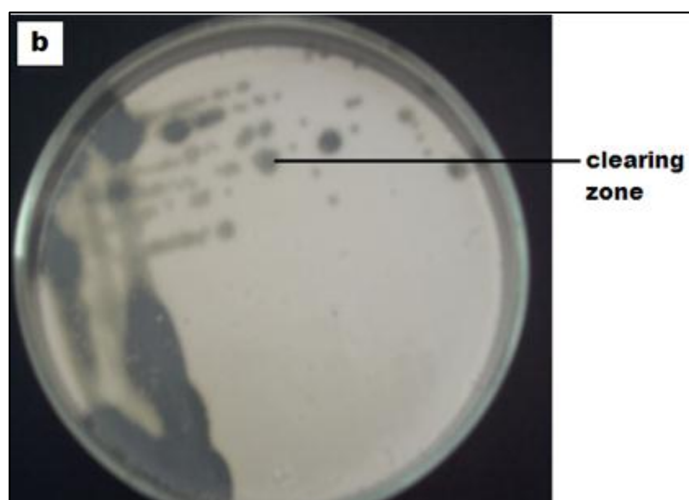

Fig 2b: Image of Acetobacter pasteurianus HBB6 colonies on calcium carbonate agar containing glucose as an energy source.

A neighbour-joining phylogenetic tree was constructed with MEGA 5.0 from partial (1100 bp) 16S rDNA sequence of the HBB6 isolate (Fig. 3). The ClustalW program in MEGA 5.0 was used to align the sequences. Sequence data from the 16S rDNA gene was submitted to BLAST at GenBank to find similar nucleic acid sequences. The HBB6 isolate had 100 % homology with Acetobacter pasteurianus. The 16S rRNA A. pasteurianus HBB6 gene sequence has been deposited in GenBank under accession number FJ998291.

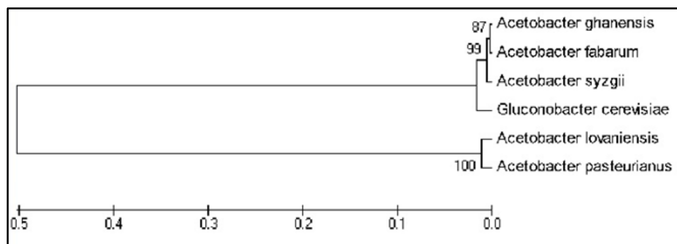

Fig 3: A neighbour-joining phylogenetic tree was constructed by MEGA 5.0. The tree shows the relationships of Acetobacter pasteurianus HBB6. Bootstrap values obtained with 1000 replicates are indicated at branching points.

3.2 Cellulose production and purification

Acetobacter pasteurianus HBB6 formed a cellulose layer on the HS medium at the end of 7 days incubation at 30oC (Fig. 4a). After purification cellulose was dried at 80oC (Fig. 4b, c).

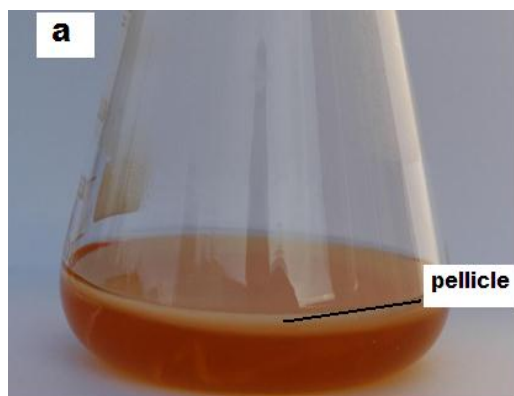

Fig 4a: Image of cellulose layer formed on the HS medium.

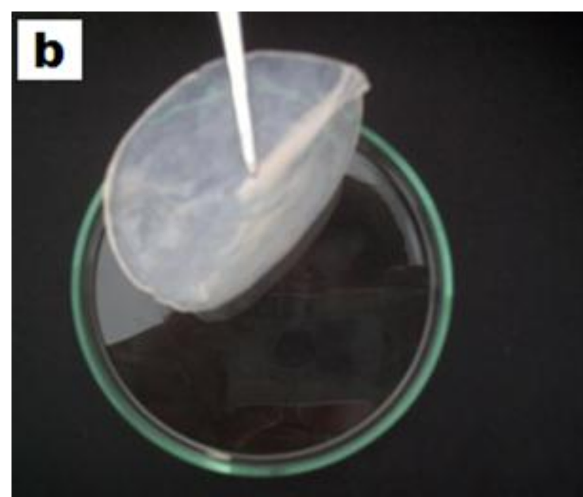

Fig 4b: Image of purified wet cellulose layer c Image of dried cellulose layer.

3.3 Effects of carbon, nitrogen sources and industrial wastes on cellulose production

The cellulose production of A. pasteurianus HBB6 strain for the various carbon and nitrogen sources used are shown in Table 1.

Table 1: Effect of different carbon and nitrogen sources on the dry weight of cells or cellulose produced by Acetobacter pasteurianus HBB6. Cells were cultivated in a static flask at 30oC for 7 days.

Medium composition	Dry weight of cell (g/l)	Polysaccharide quantify according to standard curve at 470 nm (g/l)	Dry weight of raw Cellulose (g/l)
Glucose + Yeast extract (HS medium)	0.25	2443	0.45
Glucose + Casein hydrolysate	0.26	1807	0.40
Glucose + Ammonium sulphate	0.27	1543	0.36
Sucrose + Yeast extract	0.23	1414	0.33
Sucrose + Casein hydrolysate	0.20	1241	0.25
Sucrose + Ammonium sulphate	0.12	921	0.14
Fructose + Yeast extract	0.26	2171	0.43
Fructose + Casein hydrolysate	0.33	1650	0.39
Fructose + Ammonium sulphate	0.26	1220	0.24
Ethanol + Yeast extract	0.29	1257	0.27
Ethanol + Casein hydrolysate	0.30	1214	0.20
Ethanol + Ammonium sulphate	0.18	7050	0.10

Four different carbon sources as glucose, sucrose, fructose and ethanol were used for cellulose production by Acetobacter pasteurianus HBB6. Among these carbon sources, glucose was found to be the most effective (0.45 g/l). The cellulose yield obtained was 0.43 g/l from fructose-containing medium. In sucrose-containing medium, the cellulose production was 0.33 g/l. In ethanol-containing medium the cellulose output was found to be the lowest (0.27 g/l).

In addition to the carbon sources nitrogen sources were also tested. Yeast extract, casein hydrolysate and ammonium sulphate were used as nitrogen sources. Among the used nitrogen sources, yeast extract was found to be the best nitrogen source (0.45 g/l). In casein hydrolysate medium, the cellulose yield was 0.40 g/l. In ammonium sulphate medium, the cellulose yield was found to be 0.36 g/l.

Molasses, corn steep liquor, whey and olive mill wastewater as industrial food wastes were used for cellulose production by A. pasteurianus HBB6 (Table 2).

Table 2: Cellulose production on sources molasses, corn steep liquor (CSL), Whey.

Medium composition	Dry weight of cell (g/l)	Polysaccharide quantify according to standard curve at 470 nm (g/l)	Dry weight of raw Cellulose (g/l)
Molasses	0.32	1432	0.31
CSL	0.31	1241	0.25
Whey	0.26	5060	0.16

Highest cellulose production was found on molasses (0.31 g/l). Corn steep liquor medium 0.25 g/l produced of cellulose and whey medium produced 0.16 g/l cellulose. Olive mill waste water showed no cellulose production (Table 2).

It was found that glucose and yeast extract are the best substrates among the carbon and nitrogen sources used for the production of cellulose. In addition molasses, as an industrial waste, provided the best production for cellulose.

Some studies showed that glucose, fructose, sucrose, glycerol, mannitol and ethanol were preferred as carbon sources because cellulose production by Acetobacter sp. depends on usage of aforementioned different carbon sources [6, 15, 16, 23, 24, 25, 26, 27].

Different carbon sources were tested for cellulose production from Acetobacter aceti MTCC 2623. Best carbon sources were found mannitol (1.8 g/l) and glucose (1.5 g/l), lowest carbon sources was found during ethanol presence (0.5 g/l) [23].

Jung *et al.* [16] showed that Acetobacter sp. V6 was producing 3.8 times more cellulose on glycerol than glucose containing medium. A research showed that Acetobacter lovaniensis HBB5 produces cellulose best on glucose and then fructose and sucrose. Lowest cellulose production was observed on ethanol [15]. Ruka *et al.* [25] used mannitol as carbon source and was produced 5.37 g/l cellulose by G. xylinus ATCC 53524. Raghunathan [26] showed mannitol is the best carbon source for cellulose production by Acetobacter sp. (1.38 g/l).

Dayal *et al.* [27] investigated cellulose production of Acetobacter aceti MTCC 2623 on media which contained different compounds. After 24 hours of incubation it was observed that cellulose production was higher in mannitol medium (0.0039 g/l) than glucose medium (0.0032 g/l). Santos *et al.* [6] researched cellulose production of Gluconacetobacter sucrofermentas CECT 7291 on different carbon and nitrogen sources. They found out that medium which contains mannitol (2.2 g/l) is more effective than medium which contains glucose (1.2 g/l). Among nitrogen sources most effective sources were yeast extract and corn steep liquor (2.7g/l).

Considering the articles above, it can be understood that best cellulose production of Acetobacter sp. was on mannitol and glycerol. Other tested carbon sources has (glucose, fructose, sucrose) lower cellulose production amount. Based on the carbon sources tested, change in cellulose production amount become dissimilar in metabolic cellulose synthesis pathways. For example, Acetobacter sp. oxides glucose to gluconic acid with changing pH and this inhibits cellulose synthesis.

Recently, studies on cellulose production were performed in alcohol-containing mediums [6, 8, 28]. Ethanol is a major carbon source among alcohols. The ethanol concentration in the medium influences cellulose production. Rani and Appaiah [28] used 8-10 % corn steep liquor, 0.2 % urea, 0.5-1.5 % ethyl alcohol and 1.13-1.0 % acetic acid and coffee cherry husk extract added mediums for cellulose production of Gluconacetobacter hansenii UAC09. They observed 5.6-8.2 g/l of cellulose production. Santos *et al.* [6] researched effect of ethanol on cellulose production using Gluconacetobacter sucrofermentas CECT 7291. They found 1% ethanol addition was improves cellulose production. Hyun *et al.* [8] showed that addition of alcohol (1 %) significantly increases both

microbial growth (from 0.43 to 0.81 g/l) and cellulose production (0.48 to 1.52 g/l) but 1.5-2% alcohol amount decreases cellulose production.

Considering literatures, importance of ethanol on cellulose production can be understood. If ethanol concentration is over 1 %, it causes gluconic acid accumulation and lowers the pH. This situation inhibits both microbial growth and cellulose production. 2 % ethanol that tested has decreased cellulose production amount.

In various studies, different nitrogen sources such as yeast extract, casein hydrolyzate, amonium sulphate, corn steep liquor, and peptone were investigated for cellulose production. According to these studies, the best nitrogen sources were accepted to be yeast extract and corn steep liquor [7, 16, 29]. Because of high nitrogen composites, yeast extract is an important substrate for cellulose production. The result supports the results of previous studies.

Industrial food wastes as molassess, waste beer yeast, whey and olive mill residue were tested by some researches as growth medium for cellulose production. Bacterial cellulose production (from 1.53 g/l to 3.12 g/l) was carried out using molasses and CSL by Gluconacetobacter sp. V6 [16]. Gomes *et al.* [7] showed cellulose production (2.5 g/l) using dry olive mill residue (hydrolysis with H2SO4 1M) by Gluconacetobacter sacchari. Lin *et al.* [29] investigated cellulose production of Gluconacetobacter hansenii CGMCC 3917 on waste beer yeast. Non-pretreated waste beer yeast medium 1.21 g/l of cellulose was found. However pre-treated waste beer yeast medium produced six times higher cellulose.

3.4 SEM images of cellulose

The purified pellicles were lyophilised. An SEM image of cellulose was shown in Figure 5.

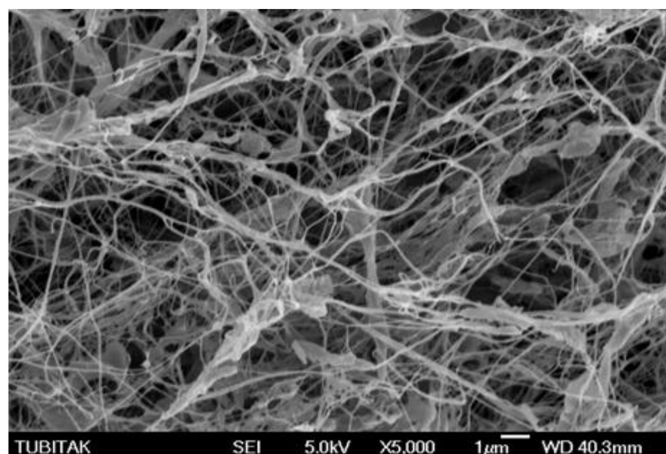

Fig 5: Scanning electron micrographs of the nanofibril structure of bacterial cellulose (magnification x 5.000).

This image shows the structure of the three-dimensional porous network of the cellulose. The ultrafine fibril structure of cellulose is not changed according to the carbon source used [24]. Many researchers reported a network of ultrafine cellulose produced by the Acetobacter species using SEM [30, 31].

3.5 Determination of the sugar composition in cellulose

The classification of cellulose as a monosaccharide was based on the products of cellulose hydrolysis. To examine the composition of purified cellulose, TLC was performed after acid-hydrolysis with 2 N TFA as described in the Research Methods. As shown in Figure 6, there was one detectable sugar in the enzymatic and acid-hydrolysis products of purified cellulose from the Acetobacter pasteurianus HBB6 strain.

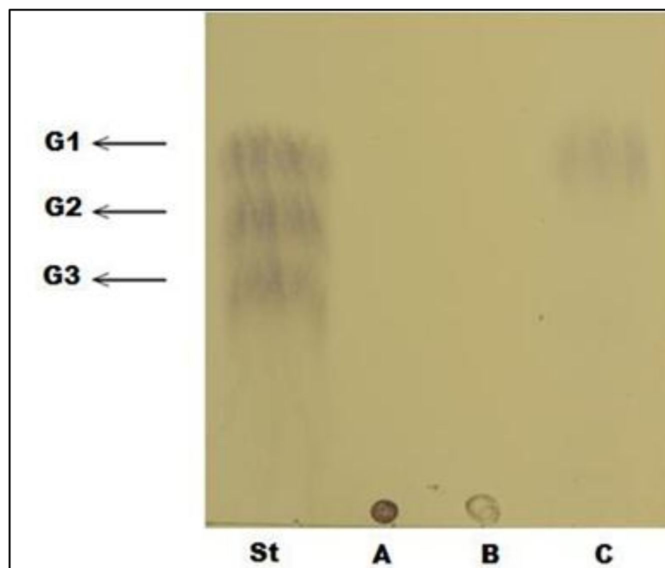

Fig 6: Thin-layer chromatography of the reaction products from the purification of cellulose from the A. pasteurianus HBB6 strain. A mixture of glucose and the cellooligosaccharides (St) Cellotriose (G3) Cellobiose (G2) Glucose (G1) Unhydrolysedavicel cellulose (A) Unhydrolysed bacterial cellulose (B) Hydrolysed bacterial cellulose with TFA (C)

TLC result showed similarity with our previous study [15]. It was proved that bacterial cellulose consists of the glucose monomers with TLC method.

3.6 FT-IR spectroscopy

Conformational properties of bacterial cellulose produced from glucose media were determined by FT-IR spectroscopy (Fig. 7). In the FT-IR spectra of cellulose the broad medium intensity band appearing at ~3412 cm-1 was assigned based on its characteristic OH absorption. Another characteristic band (CH2) was at approximately 2922 cm−1, C-O-C bounds were at approximately 1058 cm−1. These spectra of standard cellulose showed similarity with previous studies [32, 33] Luo *et al.* [32] showed that hydroxyl group bonding is found at wavelengths at 3200–3500 cm−1. Çakar *et al.* [33] indicated that the major stretching peak associated with –OH groups on the glucose molecules occur between 3230 cm-1 and 3455 cm-1 in bacterial cellulose.

The C-H stretching band of cellulose was not observed in the FT-IR spectra likely due to overlapping with the intermolecular hydrogen-bonded OH stretching frequency.

In addition, another characteristic band at approximately 1150-600 cm-1 was attributed to antisymmetric bridge stretching for the C-O-C groups in cellulose. These values are in accordance with previously reported bacterial cellulose [7, 15, 21, 31]. FT-IR result was consistent with the result reported in previous literature above.

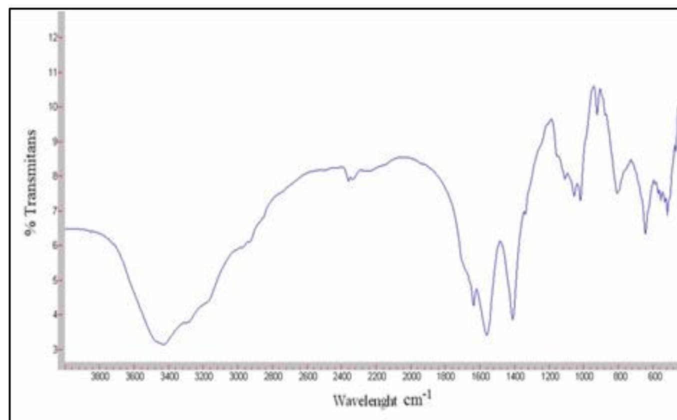

Fig 7: FT-IR spectroscopy of bacterial cellulose.

3.7 CP/MAS 13C NMR analysis

CP/MAS 13C NMR spectra of cellulose are shown in Figure 8. Analysis of purified cellulose by NMR revealed that it displays six characteristic peaks, indicating that the monomeric structure of a glucose molecule consists of six carbon atoms.

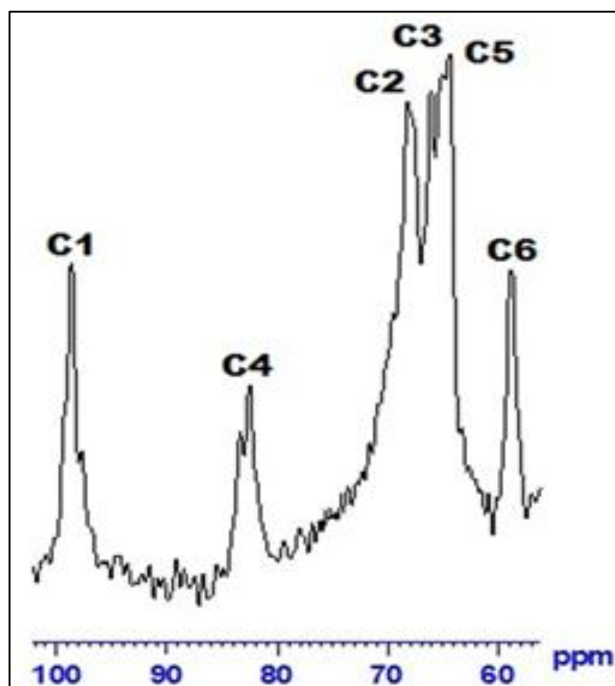

Fig 8: 13C NMR spectra of bacterial cellulose.

Results supported the findings of different studies [15, 24]. Castro *et al*. [34] reported a resonance line for C1 at 102-108 ppm, the C4 at 81 - 93 ppm and C6 at 60–70 ppm for the cellulose biosynthesized by Gluconacetobacter swingsii sp. Coban and Biyik [15] reported resonance lines for the C-1, C-4, C-2,3,5, and C-6 carbons of cellulose produced by Acetobacter lovaniensis HBB5. Furthermore, Jahan *et al*. [24] examined the 13C NMR spectra of cellulose produced by Gluconacetobacter sp. F6 and revealed characteristic peaks with regards to the six carbon atoms of glucose, which are the monomeric units of cellulose.

It was showed that a resonance line for C1 at 100-98 ppm, the C4 at 85 - 80 ppm and C6 at 60–55 ppm for the cellulose biosynthesized by Acetobacter pasteurianus HBB6.

4. Conclusion

A new strain was Acetobacter pasteurianus HBB6 from home-made wine in Turkey. It was examined different carbon, nitrogen sources and industrial wastes for optimal cellulose production. Among the different carbon sources tested, glucose was the best carbon source for cellulose production. In addition, among the different nitrogen sources tested, yeast extract was the best nitrogen source for cellulose production. However, other carbon (fructose, sucrose, and ethanol) and nitrogen (casein hydrolysate and ammonium sulphate) sources had lower bacterial cellulose production. Results of research were similar to the results of previous studies. In addition, among the industrial wastes tested, molasses was the best source for cellulose production. While, whey showed lowest cellulose production, olive mill wastewater showed no production.

Both plant and bacterial cellulose are non-branched polimer with D-glucopiranose units bonded with β-1, 4 bonds. Similarity of both celluloses can be defined with FT-IR ve CP/MAS 13C NMR spectra. Therefore, the FT-IR and CP/MAS 13C NMR spectra displayed characteristic bands and a monomeric structure for glucose molecules in a cellulose polymer. Glucose monomers in cellulose were shown with TLC. In addition, the porous structure network of cellulose was imaged with SEM.

5. Acknowledgment

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