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Production of Bacterial Cellulose by using *Acetobacter xylinum* Isolated from Local Vinegar

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Abstract

In this present research, acetic acid bacteria were isolated from local vinegar samples produced from fermented apple, date and grape; from all (26) vinegar samples; twenty-one isolates of the bacteria were obtained as a dense and smooth colonies with creamy colour on the surface of HS-agar medium, four isolates were identified as *Acetobacter xylinum* by followed many physiological and biochemical tests, the isolates were gram negative, oxidase negative and catalase positive, the isolates showed positive growth at (25, 30 and 40)°C and furthermore at pH 7.0 and 4.5, but there was no growth at (45°C), pH (2.5 and 8.5). All isolated bacteria were unable to liquefy gelatin. Four isolates were capable to ferment glucose, xylose, galactose, mannose and unable to ferment lactose, mannitol and maltose.The isolates BS2, BS3, BS8 and BS20 had ability for bacterial cellulose production. The percentage of dry weight of cellulose ranged between (2.163 – 7.234)%. Since BS2 showed the best productivity, which had the maximum cellulose production (7.234g/L) was obtained after incubation time of 7 days with Hestrin and Hchramm (HS) media in static fermentation. The isolates (BS2, BS3, BS8 and BS20) were examined for bacterial cellulose production in HS broth medium. The dry weight of crude cellulose produced by each isolates was measured and ranged from (0.36-0.42) gm. and the pH value of bacterial cellulose were (6.2-6.9), approximately equal and nearly to the neutral values with comparison with plant cellulose. The thickness of bacterial cellulose membrane is a key parameter in preparing film, **t**he initial thickness of the wet BC membrane was measured as 32 micrometers and after drying the computed thickness of BC membrane decreased to 0.4 µm. The average tensile strength value and the average elongation at break value of the dried BC films were 34.5 MPa and 5.2% respectively.

Key Words: *Acetobacter xylinum*, Bacterial Cellulose, Vinegar, GYC, Physical properties

Introduction

Cellulose is an important material for a number of industrial, several studies have documented that microbial cellulose was the alternate source. Apart from plants, various types of microorganisms also possess the ability to synthesize cellulose (Gea *et al*., 2011). Microbial cellulose is an extracellular polysaccharide that it was synthesized via various species of bacteria, including gram negative bacterial species such as *Acetobacter* sp., *Aerobacter* sp., *Achromobacter* sp., *Alacaligenus* sp., *Azotobacter* sp., *Pseudomonas* sp., *Agrobacterium* sp., *Salmonella* sp. *Rhizobium* sp., *Escherichia coli* (Zogaj *et al*., 2001 and Dahman *et al*., 2010). Cyanobacteria, and also gram positive bacteria *Sarcina ventriculi* have the ability to produce the cellulose

(Chawla *et al*., 2009), Many attempts have been made to isolate *Gluconacetobacter* sp. from fermented foods, flowers, honey, beverages, fruits, and vinegar soil and water (Maal *et al.,* 2010 and Sharafi *et al.,* 2010). Even through many more bacteria have been confirmed to produce bacterial cellulose, some spices of *Gluconacetobacter* are known to have the maximum cellulose production capacity. Production of cellulose from *Acetobacter xylinum* was first stated in 1886 by Brown, who identified a jelly like translucent mass formed over the surface of liquid fermentation medium; mother of vinegar (Czaja *et al*, 2006). It is gram-negative; obligate aerobic and rod to oval shaped (Klawpiyapamornkun *et al*., 2015), bacterial cellulose can be produced by two main techniques including static culture (Kralisch *et al*., 2010) agitating culture (Tse *et al*., 2010). The choice of cultivation methods is dependent on further bio-cellulose commercial destination. Each technique has advantages and disadvantages (Lee *et al*., 2014 and Gromovykh *et al*., 2017). One of the most advanced kinds' non- photosynthetic bacterium can utilize glucose, glycerol or other organic substrates and turn them into pure cellulose (Brown *et al.,* 1976 and Yamada *et al.,* 1997).

This organism within the family *Acetobacteraceae* have unique in productive synthesis, large quantity of high quality cellulose organized in the form of a single, twisted, interwoven extracellular ribbon as part of the primary metabolite, the typical cell of A. *xylinum* can polymerize up to 10^8 glucose molecules per hour into cellulose (Battard-Bernardo *et al.,* 2004). Generally, structure of microbial cellulose and plant cellulose, are chemically the same, in the form of β-1,4-glucans, both of them have the same molecular formula as $(C_6H10O_5)n$, but varies from chemical and physical features (Santos *et al*., 2015). The production of this BC, is receiving great interests due to its superior properties like free of contaminating hemicellulose and lignin, it can be purified more easily than plant cellulose and its cryctallinity and degree of polymerization are higher compared to plant cellulose (Auta *et al*., 2017), because of its greater mechanical strength, water holding capacity, in-situ moldability and larger surface area, insolubility in most of the solvent, biocompatibility and biodegradability, elasticity, non-toxic with no allergenic side effects materials (Pecoraro *et al*., 2008; Donini *et al*., 2010 and Wei *et al*., 2011).

Bacterial cellulose has been used in multifarious application such as "Kombucha tea" as dietary drink and traditional popular desert "nata de coco". Bacterial cellulose moreover used as a binding, coating, thickening and emulsifying agent in food industries (Chawla *et al*., 2009), textile and paper industry (Weber *et al*., 2002) pharmaceuticals and medical applications, optically transparent composites (Nogi and Yano, 2008), drug delivery systems (Singh *et al*., 2016), in the manufacture of audio products such as headphone diaphragms (Hassan *et al*., 2015). HS media (Schramm and Hestrin, 1954) which composed of D-glucose, citric acid, disodium phosphate, yeast extract, and peptone and distilled water (Son *et al*., 2001), an optimum growth temperature is ranging between (25-30)°C, suitable pH values ranging from 4.5 to 6.2 and duration of cultivation from (3-7) days the production efficiency is usually about 50%. (Esa *et al*., 2014). Cellulose biosynthesis in *A. xylinum* happens between the outer and plasma membranes by a cellulose-synthesizing complex, which is in association with pores located in the surface of the bacterium (Cannon and Anderson, 1991 and Mehta *et al*., 2015). The present study, aimed to determining the physical properties of BC produced by *Acetobacter xylinum* isolates. The physical analysis includes, thermal stability using thermogravimetric analysis (TGA), pH, moisture

content, compressibility index and swelling index. The properties of BC had been compared with the plant cellulose products powder (BC).

Materials and Methods

Sample collection

Fermented fruit samples (vinegar) collected from August 2016 to March 2017, twenty-six local vinegar samples (apple, date and grape) was collected from various places of Baaqubaa and Erbil cities, each sample were placed into sterile plastic containers and transported to the laboratory. The samples were examined separately and in triplicate for each sample.

Sample preparation and Isolation of cellulose producing-bacteria

One ml of each vinegar sample was taken and added to the Erlenmeyer flask containing 30 ml of standard HS-medium broth, and incubated at 30°C for (7-10) days, then a loop full of cell growth pellicle was streaked on the HS agar plates, incubated at 30°C for 72 h. All cultures were purified, and then streaked on the Glucose Yeast Calcium Agar (GYCA) medium to verify acid production. Colonies which produced clear zone were subjected to morphological, culture and biochemical tests.

Identification of Bacterial cellulose isolates:

Morphological, physiological and biochemical tests were performed for identification of bacterial isolates colony shape, color and size of each bacterial isolates were examined on CaCO3 medium after incubation at 30Cº for 3 days; this could be a tool for the preliminary identification (kadere *et al.*, 2008). Biochemical test; catalase, oxidase, indole, Triple Sugar Iron Agar (TSI agar), urease, gelatinase, motility, Simon citrate and carbohydrates test were done for identification also the isolated cultured on glutamate media and on mannitol media (Aydin and Aksoy, 2009), as well as GYP agar (Drysdale and Fleet, 1988), Glycerol ketogenesis test and Alcohol oxidation test by culturing on Carr medium (Klawpiyapamornkun, *et al*., 2015).

Production of Bacterial cellulose:

The static cultivation method used in this study for the production of BC process, 10 ml of the bacterial culture was added to 90 ml of HS medium in a 500 ml flask (10^6 CFU/ml) and incubated at 30Cº for maximal 10 days statically. The bacterial cellulose with highest cellulose production was selected.

Preparation of BC powder

The BC pellicle obtained was washed thoroughly with water. Then it was saturated and treated with boiling water at 100°C for 1 hr. using a water bath. Then the BC pellicle was crushed using a heavy duty blender and poured into a tray. It was dried in the oven at 50°C for 24 hr. The dried BC was then ground again until a fine BC powder was obtained (Halib *et al.*, 2012).

Swelling properties

Two grams (2 g) of BC, and PC samples were mixed with 40 g of different solution separately (distilled water, acetone, dimethyl sulfoxide (DMSO) and acetic acid). The suspension was allowed to stand for 2 hr. The suspensions were centrifuged at 3500 rpm for 30 min. The supernatant was removed and the wet precipitate was weighed. The steps were repeated three times to get the average

reading (Goh *et al.*, 2012). The result was expressed as a gram of water per gram of sample (w/w) by using Eq. 1.

Liquid retention level = M1/M2 - 1 × 100 (1)

Where, M1 = weight of wet sample and M2 = weight of dry sample.

Determination of pH

One gram of BC andPC samples were mixed with 40 ml of distilled water at pH 6.8 separately. The solution was stirred for 15 min using a hot plate and magnetic stirrer. The pH was checked using the pH meter and the reading was recorded. The steps were repeated three times and the average reading was calculated (Halib *et al.*, 2012).

Determination of moisture content

Five grams of BC and PC samples were accurately weighted separately. Then, the samples were placed in the moisture analyzer and the reading was recorded. The steps were repeated three times and the average reading was calculated (Azubuike and Okhamafe, 2012).

Results and Discussion

Isolation and Identification of *Acetobacter xylinus*

The bacteria were isolated from twenty six local vinegar samples; twenty-one isolates were obtained; appeared dense and smooth colonies with creamy colour were observed on the surface of HS-agar medium. Suspected colonies were purified by culturing onto GYC media to confirm acetic acid production. Only four isolates showed transparent zone around the colony on GYC agar plates as shown in figure 1, these colonies were belonged to acetic acid bacteria as mentioned by Lisdiyant *et al*., (2003) as well as the characters of these isolates were closely associated to acetic acid bacteria as revealed by Yamada *et al*. (1999). In addition, all four isolates (BS2, BS3, BS8, and BS20) were gram negative, ellipsoidal to rod shape and single, paired or chain appeared when examined by microscope, these results agree with the findings of other studies (Zahoor *et al*., 2006; Brenner *et al*., 2005; Son *et al*., 2002; and Lisdiyanti *et al*., 2001), also similar results were obtained by Kowser *et al*., (2015) and Aydin and Aksoy (2010).

The biochemical tests for isolated bacteria were examined, the results shown in the table (1), the isolates BS2, BS3, BS8 and BS20 motile, oxidase negative and catalase positive. A negative reaction was observed for urease, gelatines liquefaction, H_2S formation, indole production, and glutamate also citrate utilization. On the other hand ketogenesis of glycerol for three (BS2, BS3 and BS8) isolate were positive, but the isolates BS20 gave negative results. These characters mentioned above; the same in Bergey's manual (Holt *et al*., 1994). All the isolates were grown, at 30C^o, but weak at 25C^o, while at 45C^o was no any growth. Isolates showed growth at pH 4.5, 6 and 7, while no growth occurred at pH 2.5 and 8. All the isolates fermented the testing sugars; glucose, xylose, galactose, mannose with the production of acid and gas,

whereas they were unable to ferment; lactose, fructose, maltose and mannitol. The isolate B2 distinguished from others by acid and gas formation from sucrose also B8 was fermented fructose and producing acid and gas as appeared in table (1), this was previously recorded by numerous studies (Mounir *et al*., 2016; Aydin and Aksoy, 2010; Kadere *et al*., 2008 and Son *et al*., 2002).

Acetobacter xylinum had the ability to fermented most of the carbohydrates. The isolates could oxidize acetate and lactate into CO_2 and H_2O, and oxidized ethanol to acetic acid and further incubation, the acetic acid was completely over-oxidized to CO_2 and H_2O; the isolates altered the medium colour from green to yellow and then reverted to the green colour as appeared in figure 2. Therefore, these isolates belong to Acetobacter species (phong *et al*., 2017; Mukadam *et al*., 2016; Moryadee and Pathom, 2008; and Sokollek *et al*., 1998), all the isolates were unable to produce brown pigmentation on GYP- agar plates.

Table (1): Physiological and Biochemical Tests for *Acetobacter xylinum* Isolates.

Biochemical test	BS2	BS3	BS8	BS20
Motility	+	+	+	+
Oxidase	–	–	–	–
Catalase	+	+	+	+
Urease	–	–	–	–
Gelatin liquefaction	–	–	–	–
H2S formation	–	–	–	–
Indole production	–	–	–	–
Growth on glutamate agar	–	–	–	–
Citrate utilization	–	–	–	–
ketogenesis of glycerol	+	+	+	–
Oxidize acetate	+	+	+	–
Oxidize lactate	+	+	–	–
Over-oxidation of acetic acid	+	+	–	–
Oxidation of ethanol	+	+	+	+
Brown pigmentation	–	–	–	–

Cellulose production	+	+	+	+
Acid and gas production from				
Glucose	+	+	+	+
Xylose	+	+	+	+
Galactose	+	+	+	+
Mannose	+	+	+	+
Lactose	–	–	–	–
Fructose	–	–	+	–
Maltose	–	–	–	–
Mannitol	–	–	–	–
Sucrose	+	–	–	–
Growth at different temperatures				
25°C	W	W	W	W
30°C	+	+	+	+
45°C	–	–	–	–
Growth at different pH				
2.5	–	–	–	–
4.5	+	+	+	+
6	+	+	+	+
7	+	+	+	+
8	–	–	–	–

(+): Positive Result; (-): Negative Result; (w) weak Result

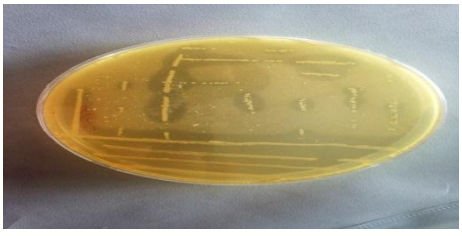

Figure 1: The Clear Zone around the Colony of *Acetobacter xylinum* on GYC agar plates.

Figure 2: *Acetobacter xylinum* on Carr Medium.

Screening of Local *Acetobacter xylinum* for Cellulose Production

The ability of the isolates BS2, BS3, BS8 and BS20 were examined for bacterial cellulose production in the HS broth medium. The dry weight of crude cellulose produced by each isolates was measured, the results appeared in table (2) that all of the isolates produced cellulose, the formation of white pellicle near the surface of the static culture medium as shown in figures (3,4,5). The percentage dry weight of cellulose ranged from 2.163 – 7.234 %. Since BS2 showed the best productivity, which had the maximum cellulose production, on the bases of this the isolate BS2 was selected and using in further studies. The yield percentage of BC calculated according the following equation:

$$\text{BC Content (\%)} = \frac{\text{BC (g/l)} \times 100}{\text{Cell dry weight (g/l)}}$$

The differences in cellulose production amount were due to variation to pyrophosphorylase activity, which has an important role in cellulose biosynthesis, and due to the effect of environmental circumstances (Bielecki *et al*., 2005).

Table (2): The Percentage of Bacterial Cellulose Production

Isolate	Source of isolates	Cellulose weight (g/L)
BS2	Apple vinegar	7.234 %
BS3	Apple vinegar	5.122
BS8	Date vinegar	3.567
BS20	grape vinegar	2.163

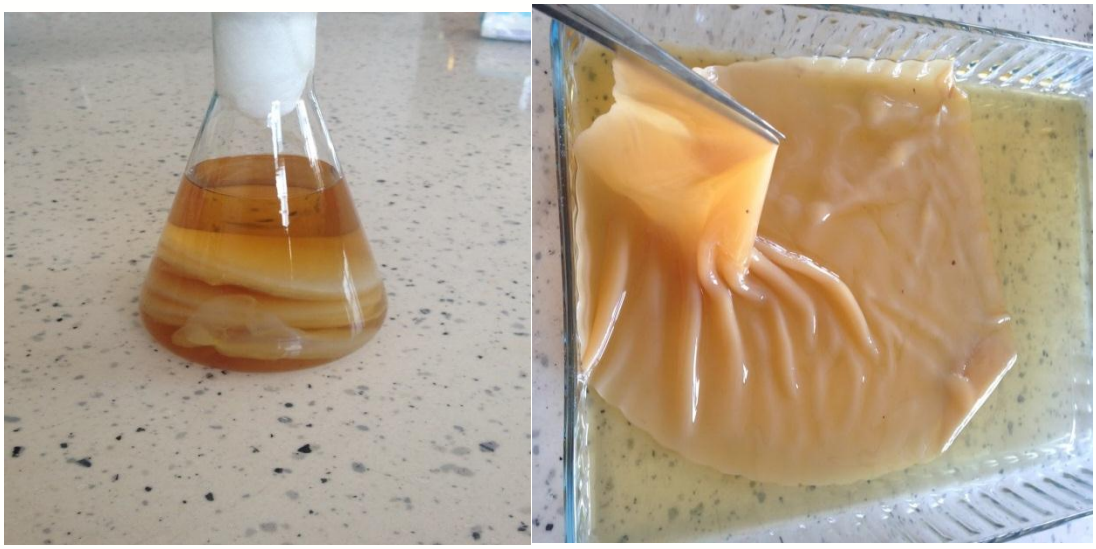

Figure 3: formation BC film in HS broth medium.

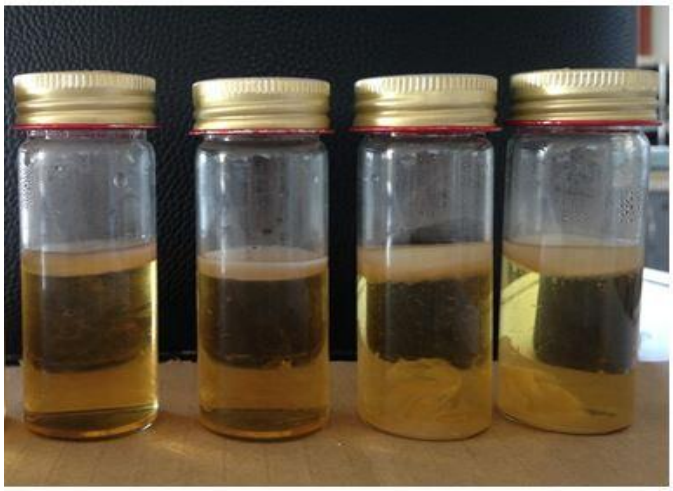

Figure 4**:** Surface compact disk of Bacterial Cellulose Formed By *Acetobacter Xylinum* Isolates

Figure 5: Purified of Bacterial Cellulose Film Formed By *Acetobacter Xylinum.*

Physical properties of the bacterial cellulose:

pH Value

The pH value of BC and PC samples were 6.2 and 6.9 respectively. These results are consistent with the British Pharmacopoeia (United States Pharmacopaea 2004 and British Pharmacopoeia,2004). The prepared both bacterial and plant cellulose powder's pH during this study was within this description.

Thickness measurement

The thickness of bacterial cellulose membrane is a key parameter in preparing film, **t**he initial thickness of the wet BC membrane was measured as 31-32 micrometers and after drying the computed thickness of BC membrane was decreased to 0.4 micrometers as shown in table (3). The difference between BC membrane thickness measures can be due to several factors, such as incubation time, culture temperature and medium conditions. Tang *et al.*, (2010) has mentioned that during cultivation, the yield of BC and cell mass increased with time and accumulation of more fibrils.

Mechanical performance

The mechanical properties, which consist of the tensile strength and elongation at break of the BC films with an average thickness of (0.4) µm were shown in table (3), the average tensile strength value and the average elongation at break value of the dried BC films were 34.5 MPa and 5.2% respectively. Results indicated that the BC films produced by *Acetobacter xylinum* had high strength; this due to the powerful H-bonds among cellulose molecules and high crystallinity led to the highest strength and tensile strength of BC films. These results agree with the findings of other studies, but

overall the values regarding the parameters of the mechanical properties differ according to the methodology followed. Moreover, mechanical properties of BC films depended on the numerous factor, such as incubation time, failure stress under uniaxial(Keshk,2006), the drying method applied (Rani *et al.,* 2011), the concentration of NaOH used for BC treatment (Shezad *et al.,* 2010) as well as the pressure applied to the membrane before drying (Tang *et al.,* 2010).

Table 3: The Physical Properties of Local Bacterial Cellulose

Characters	Bacterial Isolates			
	BS2	BS3	BS8	BS20
Wet weight (gm) **Dry weight**	34.2 0.40	34.5 0.42	33.9 0.36	34 0.39
pH value	6.5	6.5	6.2	6.9
Wet thickness(µm) **Dry thickness(µm)**	32 0.4	31 0.4	32 0.4	32 0.4
Tensile strength(MPa) **% of elongation**	34.5 5.2	34.4 5.1	34.5 5.2	34.4 5.2
Moisture content%	8.52	8.75	8.23	8.23

Swelling and Absorption Characteristics

The water absorption capacity values of BC in comparison with plant cellulose in various type of solvent at room temperature illustrated in figure (6). Recent evidence suggests that the inter-crystalline and intracrystalline in cellulose was possible to highly swelling in specific solvents (Mantanis *et al.,* 1995). In general, BC was higher retention level in all solvents compared to PC, BC returned to the reflection of higher level of swelling characteristic of bacterial cellulose, this is because BC has three-dimensional nanofibrils network structure that can absorb higher amount of water. Several researchers reported that water retention value of BC up to 100 times of its dry weight (Czaja *et al.,* 2006; Lina *et al.,* 2011; White and Brown, 1989).

According to (Robertson 1964), the swelling power of the cellulosic fibers within the solvent was affected by the degree of molarity volume of the solvent that provided to the H- bonding ability and the cohesive energy density of the solvent.(Robertson 1964 and Cheng *et al.,* 2010).

The result additionally represented that bacterial cellulose shows the best swelling value ranging between (18 and 210%) in all the six solvents than that of PC (10 and 90%). The result during this study mentioned that; dimethyl sulfoxide resulted within the best cellulose swelling value for both the BC and PC, compared to the water. However, acetone showed the lowest cellulose swelling value. The obtained results match those observed in earlier studies (Mantanis *et al.,* 1995 and Zahan *et al.,* 2017).

The LRV in commercial and bacterial cellulose-based products is most important mainly when it was used as a binder, adhesive, thickener, encapsulating agent, fat-sparing agent, gelling agent, film former, and texturizer and with several other applications in the food and non- food products (Shi *et al.*, 2014 and Kaur *et al.*, 2011).

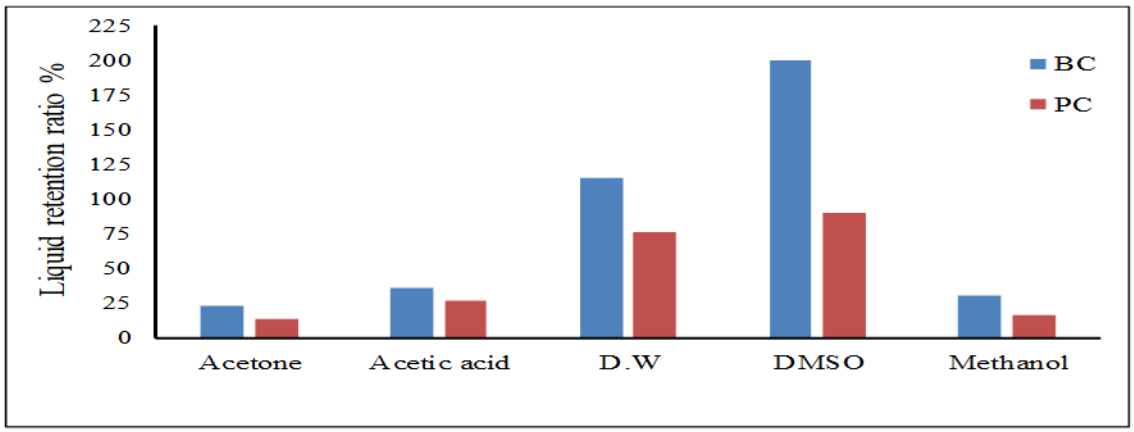

Figure 6: Swelling of bacterial cellulose and the commercial PC in five different solvent.

Conclusion

The study was concluded that the acetic acid bacteria *A. xylinum* was isolated from local vinegar samples and identified by classical methods as well as production of bacterial cellulose in HS medium and physical properties of BC were investigated. The data collected had proven that BC shows a better and promising characteristic compared to plant cellulose. In conclusion, BC can be used as one of the alternative to replace the plant based cellulose product in the industries which finally can be used in food packaging and coating of food

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