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Production of Bacterial Cellulose from *Acetobacter Xylinum* using Fruits Wastes as Substrate

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Abstract:
Bacterial cellulose (BC) are cellulose that are biologically produced from several species of Acetobacter because of its unique properties, it has advantages over plant cellulose. Acetobacter xylinum, which produces Bacterial cellulose from various substrates including sugars, fruits and vegetable wastes. Bacterial cellulose produced by Acetobacter xylinum was a new type of biopolymer. Nowadays, biopolymer has numerous applications in industrial sector and biodegradable and nontoxic in nature. In this regards a focus is given to coconut water, papaya juice and muskmelon juice (wastages) were used as substrates for Bacterial Cellulose production. The objective of this work was to produce bacterial cellulose using low cost and natural carbon sources and Comparative study between coconut water, papaya juice and muskmelon in the production of Bacterial cellulose. The design of the study employs with three treatments viz. Coconut water, muskmelon and papaya juice medium without any additional nutrients (Blank), with additional sugar (i.e. 12% glucose with 5.0% v/v Acetobacter xylinum were added to fruits juice medium) and without additional sugar (only Acetobacter xylinum added to fruits juice medium). Acetobacter xylinum (NCIM 2526) was cultured in a coconut water, papaya and muskmelon juice medium to produce BC. The maximum thickness of BC was measured on 7th day of fermentation. The highest yield of BC obtained for coconut water, papaya juice and muskmelon found to be is 2.43g/100mL, 4.52g/100mL and 1.68g/100mL i.e. without additional sugar when compared with the substrates having additional sugar. Hence papaya juice gave the highest yield using natural carbon source i.e. without additional sugar followed by coconut water and muskmelon juice gave lowest cellulose yield. Whereas there was no visible cellulose layer formed in Blank solution. Finally it is concluded that, the Cellulose production increases with use of particular carbon sources in some media, but not in others and yield is greatly affected by selection of media. It could be concluded that static condition is suitable for Bacterial cellulose production.

Keywords: *Acetobacter Xylinum, Bacterial Cellulose, Coconut water, Muskmelon juice, Papaya juice*

1. Introduction

Cellulose is most abundant natural biopolymer on the earth, synthesized by plants, algae and some species of bacteria. It is produced by some animals (e.g. tunicates) [1]. Bacterial cellulose is polysaccharide produced by *Acetobacter xylinum*, it grows in air-liquid surface of culture medium not only in a static culture, but in shaking flask and stirring bioreactor [2]. Many species of bacteria, such as those in the genera *Gluconacetobacter, Agrobacterium, Aerobacter, Azotobacter, Rhizobium, Sarcina, Salmonella, Enterobacter, Escherichia* and several species of cyanobacteria are likely to produce extracellular cellulose [3]. Among these, gram-negative *Acetobacter xylinum* is claimed to be an effective cellulose-producing bacterium and is widely used [4]. The genus *Acetobacter* refers to a group of bacteria that have the ability to oxidize sugars, sugar alcohols and ethanol producing acetic acid as major end product [5]. Cellulose-producing bacteria are commonly found in natural sources such as flowers, vegetables, nuts, sugar cane and in particular, rotten fruits [6]. The strains of *Acetobacter xylinum* are capable of producing cellulose in varying amounts and growing on a wide variety of substrates viz. glucose, sucrose, fructose, invert sugar, ethanol and glycerol, *Acetobacter xylinum* has been shown to be distinctly acid tolerable capable of growing at pH as low as 3.5 and reported pH 4.0 and 5.0 to be ideal for the development of cellulose [7]. Cellulose derived from bacterial source is called BC [3]. Bacterial cellulose can grow at many culture mediums. Coconut water is one of the culture medium usually used to produce bacterial cellulose by *Acetobacter xylinum*. It is popularly known as '*nata de coco*' with a smooth surface and chewy texture, Fermentation process using coconut water medium was implicated to produce BC by *Acetobacter xylinum* around 7-8 days in a static batch [8]. Unconventional product based on coconut water is native to Philippines and developed locally first in 1949. Over the years, it has become popular in other countries like Japan, Korea and USA [7]. BC is one of cellulose sources, which have unique properties including high mechanical strength, high crystallinity and water retention ability. Moreover, BC has high purity, free of hemicelluloses, lignin and non-cellulosic compound. It is easy to be decomposed by microorganism in environment and

renewable. Therefore, it has been used in many industrial processes such as food industry, paper industry, textiles industry, electrical application and membrane [8].

Bacterial cellulose produced from expensive culture media, containing glucose as carbon source and other nutrient sources resulting in very high production costs, which limits the use of material to a very high value added applications. The use of cheaper carbon and nutrient sources is an interesting strategy to overcome the limitation and therefore to increase the competitiveness of unique material. Mostly Fruits are sold and consumed as raw food, but most of the damaged and non-standard size ones are shelved, though some are processed to make jams, paste's and sauces. When fruits cannot be shipped because of their poor quality caused by bad weather and some other natural disasters, it leads to low prices and fruit wastages. Majority of wastes end up being discarded. However, fruits have abundant sugars such as glucose and fructose that could be bio-converted into useful products such as Bacterial cellulose [3]. Bacterial cellulose has important applications in a variety of food formulations. It is especially used when low-level use, lack of flavor interactions, foam stabilization and stability over a wide range of pH, temperature and freeze-thaw conditions are required. Potential uses include pourable and spoonable dressings, sauces and gravies, icings, sour cream, cultured dairy products and aerated desserts and frozen dairy products [9]. Bacterial cellulose has wide range of applications which include temporary artificial skin for therapy of burns, ulcers and dental implants [10]. The commercial applications for Bacterial cellulose such as "Nata" a food product of the Philippines, audio headphone diaphragm and artificial skin for scalded or wound healing [11].

The essential components of Bacterial cellulose growth media are carbon and nitrogen which will provide nutrients to growth of *Acetobacter Xylinum*. When it is compared between use of glucose and sucrose, the use of glucose produces thicker Bacterial cellulose [12]. Bacterial cellulose was produced from beet molasses using *Gluconacetobacter xylinus (*ATCC 10245). The yield of bacterial cellulose (BC) produced from beet molasses was higher than the glucose as a sole carbon source [13]. Bacterial cellulose was produced by bacterium, *Gluconacetobacter xylinus* in sucrose and date syrup solutions (Bx. 10%) under static condition at 28^0C using *G. xylinus* (PTCC, 1734). The results showed that, the maximum yields of bacterial cellulose (BC) were 4.35 and 1.69 g/l00 mL for date syrup and sucrose medium after 336 hours fermentation period [14]. Another interesting approach is to produce edible film from bacterial cellulose by using *Acetobacter Xylinum* in the media of coconut water and *Arenga pinnata* juice. Fermentation method was used to produce bacterial cellulose as edible film material. Edible film sheets were produced by *Acetobacter Xylinum* under different culture medium. The 100% of *Arenga pinnata* juice, 100% of coconut water and 75% *Arenga pinnata* juice and 25% coconut water, 25% *Arenga* 26 *pinnata* juice and 75% coconut water and 50% *Arenga pinnata* juice and 50% coconut water mixtures. The products of edible film from different culture medium tested for physical properties and observed for biodegradation in soil. The results showed that, the percentage of weight loss increased during intervals of time. Edible film yielded from the media of 25% *Arenga pinnata* juice and 75% coconut water indicated the highest of % weight loss at 9th day. The physical properties of edible film were figured out as water absorption, strength of break, and elongation. Results showed that, the edible film produced from 75% of *Arenga pinnata* juice and 25% coconut water had highest elongation around 10%. In addition, the strength of break of edible film produced from 50% of *Arenga pinnata* juice and 50% coconut water was 2.27 Kgf/mm^2. It was highest among other culture medium. The hypothesis on the study was edible film could be produced from the culture medium. Edible film can be an alternative to solve the environmental problems [8]. In another study, *Acetobacter Xylinum (*TISTR 975) was cultured in a coconut water medium with added $MgSO_4$ and NH_4HPO_4. The optimum pH and amount of added sugar for the production of cellulose found to be 4.75 and 5.1%w/v and production of cellulose measured on eighth day of fermentation, while the depth of liquid medium and surface area varied. The depth of medium for highest cellulose production found to be 2.5 cm. The increment of medium volume by depth of liquid medium was less effective for the production of cellulose than the increasing surface area. Highest amount of cellulose produced was 5.94 g/L from the fermentation conditions of 2.5 cm depth and liquid/air interface area of 359.12 cm^2 [15]. The production of Bacterial cellulose from *Gluconacetobacter persimmonis* GH-2 organism could able to use various carbohydrates (2% w/v) for growth and cellulose production. Bacterial cellulose production from various natural carbon sources provided at 2% in HS medium. All natural carbon sources were able to support growth and cellulose production by *Gluconacetobacter persimmonis*. The molasses, watermelon, orange juice and muskmelon were provided as carbon and nutrient sources, the organism was able to produce 5.75, 5.98, 6.18 and 8.08 g/L of Bacterial cellulose. The maximum Bacterial cellulose yield was given by muskmelon [3]

In India, offering coconut to god is one of the integral parts of worship. During this lot of coconut water is generated as a waste from temples. Coconut water thus discharged from temples, is rich in minerals with small amounts of sugar, proteins, vitamins and growth promoting substances and can be microbiologically processed to yield Bacterial cellulose.

The present study reported that the effect of various natural carbon sources like fruit juices (Papaya and Muskmelon juice), coconut water and additional carbon sources (i.e. with 12% Glucose) on cellulose production by *Acetobacter Xylinum.* This study was done to compare Bacterial cellulose production in different fruit juices medium. (i.e. with and without additional sugar).

2. Materials and Methods

2.1. Materials

All chemicals used in the experimental protocol are of analytical reagent (AR) grade. All chemicals were purchased from Merck chemical (Bangalore). *Acetobacter Xylinum* (NCIM 2526) Culture obtained from National Collection of Industrial Microorganisms (NCIM), Pune. Whereas the waste fruits i.e. Papaya and muskmelon collected from Local market and coconut water collected from temple.

2.2. Culture Maintenance

Acetobacter Xylinum (NCIM 2526) culture obtained from National Collection of Industrial Microorganisms (NCIM), Pune, India. The culture was maintained on HS medium with composition as mentioned by Hestrin and Schramm [16]. The composition of medium was (g/100mL): glucose (2g), yeast extract (0.5g), peptone (0.5g), citric acid (0.115g), disodium hydrogen phosphate (0.11g). The reagents were dissolved in distilled water, the volume was brought up to 100 mL and pH was adjusted to 6.0 with addition of hydrochloric acid or sodium hydroxide and sterilized at 121^0C for 15min. *A. xylinum* was streaked on these slants and incubated at 30^0C for 4 days.

2.3. Preparation of inoculum

Acetobacter xylinum grown on HS agar slants was inoculated into sterilized media containing glucose (2g), yeast extract (0.5g), peptone (0.5g), citric acid (0.115g), disodium hydrogen phosphate (0.11g). The reagents were dissolved in distilled water, the volume was brought up to 100 mL and pH was adjusted to 6.0 with addition of hydrochloric acid or sodium hydroxide and sterilized at 121^0C for 15min. The inoculated media was incubated statically at 30^0C for 4 days. Sub culturing was done to maintain the purity and viability of microorganism as shown in Figure 2.1.

Figure 2.1: Acetobacter Xylinum on Liquid Media.

2.4. Production of BC from fruit juices

In this study, Coconut water collected from temple whereas papaya and muskmelon fruit collected from local market. These fruits were washed, crushed, squeezed and separated to the juices and residues. The juices were diluted, filtered, sterilized and stored at -20°C for future use. Experiment was carried out in three stages viz. Blank (i.e. no sugar and culture added to fruits juice medium), sample C1 was kept as with additional Sugar (i.e. 12% glucose and 5.0% v/v *Acetobacter xylinum* was added to fruits juice medium) whereas sample C3 was kept as without additional sugar (i.e. only culture was added to fruits juice medium) as shown in the following Figure 2.2 (a), (b) and (c). pH of these fruits juice medium was adjusted at 5.0 with acetic acid (1%) and then the medium was boiled in a wide mouth container and cooled. Then, the strain of *Acetobacter Xylinum* 5.0 % (v/v) was inoculated into this coconut water, papaya juice and muskmelon juice medium aseptically and incubated for 15-20 days in undisturbed condition at room temperature (28-30^0C). During this period a gelatinous pellicle will be formed on the surface of the medium. The gelatinous pellicle was separated from the medium and washed thoroughly using distilled water to remove loose fibers and acid. The sample was analyzed for wet weight, dry weight, thickness, moisture content and yield of Bacterial cellulose was determined.

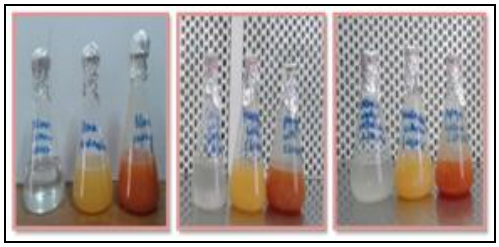

Figure 2.2: (a) Blank (b) With Sugar (c) Without Sugar

2.5. Harvesting of Bacterial Cellulose

The Bacterial Cellulose layer formed after 15–20 days was harvested when it was about 0.8–1.0 cm thick, washed repeatedly with water to remove glacial acetic acid. The layer of Bacterial cellulose was immersed in water for 24 h with repeated changing of water to remove the sour odour.

2.6. Evaluation of Bacterial Cellulose by Analytical Methods

2.6.1. Thickness of Bacterial cellulose

Thickness of each bacterial cellulose membrane was measured at ten different positions by a thickness gauge and values were averaged.

2.6.2. Determination of Wet Weight and Dry Weight Cellulose

Harvested microbial cellulose washed with NaOH solution 2% (w/v) for 30 min and thoroughly washed with distilled water thoroughly and Record the wet cellulose weight and dried at 75^0C in an oven for 6 hours, Cooled to ambient temperature and record the dry cellulose weight. The dry weight of the cellulose obtained was calculated.

2.6.3. Moisture content of Bacterial Cellulose

The moisture content (%w/w) of bacterial cellulose was determined based on the weight loss of bacterial cellulose when dried at 75^0C.

$$\text{Moisture content \%} = \frac{(\text{wet weight} - \text{Dry weight}) \times 100}{\text{Dry weight}}$$

2.6.4. Percent yield of BC

Percent yield of BC was calculated by following Equation

$$\text{Percent yield} = \frac{\text{Dry weight of BC} \times 100}{\text{Weight of carbon source used in Production medium}}$$

2.6.5. Determination of Sugar Content of Bacterial Cellulose

The 10mL of sample is taken and centrifuged at 5000 rpm for 3 minutes to sediment the cellulose and others solutes. 3mL of supernatants were carried out and added with 3mL of 3, 5-dinitrocylicsilic (DNS) and centrifuged thoroughly. The mixing is placed into water bath for 90^0C at 15minutes. After cooled to ambient temperature 1.0mL of potassium natrium tartarate was pipette into the solution for color stabilizing. Cell cuvet fill with the mixing solution and read the optical density (OD) at 550nm wavelength.

3. Results and Discussion

To meet the objectives of the project work, a detailed discussion are discussed in the subsequent section.

3.1. Bacterial Cellulose

The cultures (*Acetobacter Xylinum*) were inoculated in coconut water medium and papaya juice medium, visible clouds of cellulose began to form within one to two days. A thick skin of cellulose formed on the surface of media within 5-7 days. *Acetobacter Xylinum* strain yielded a pure form of cellulose that was transparent and slippery as shown in Figure 3.1. The Figure 3.2 shows the bacteria cellulose produce in papaya juice medium. The bacterial cellulose harvested from papaya juice medium after 15 to 20 days of fermentation are shown in Figure 3.3.

Figure 3.1: (a) Photographic View of Synthesis of Bacterial Cellulose in Coconut Water Medium (b) Top View of Bacterial Cellulose Produced at the Air-Liquid Interface

Figure 3.2: Bacterial Cellulose Produced in Papaya Juice Medium
Figure 3.3: Bacterial Cellulose after Harvesting from Pa-paya Juice Medium

3.2. Bacterial Cellulose Production in Static Culture Condition

Table 3.1 and Figure 3.4 showed that, the measurement of Bacterial cellulose thickness with respect to time and sugar concentration. In the first day of cultivation there was no cellulose production, turbidity of the culture broth increased within a day of inoculation and a cellulosic film was formed on air-liquid interface at room temperature. Incubation was lasted for week and cellulose production was monitored and with respect to time, cellulose production increases. The Figure 3.3 and Figure 3.4, it could be clearly observed that, the three variations showed cellulose fiber formation results from 1st day to the 7th day of fermentation. The maximum thickness of Bacterial cellulose i.e. without additional sugar was observed on the 7th day of fermentation in coconut water, papaya juice and muskmelon was 11mm, 14mm and 8mm hence Papaya juice medium without additional sugar gave maximum thickness when compared with other substrates. Hence Papaya juice medium without additional

sugar gave maximum thickness when compared with other substrates since as sugar concentration increases, cellulose production decreases. Therefore, an appropriate level of sugar is necessary for optimum bacterial cellulose production.

Time (hrs)	Thickness in mm		
	Coconut (without sugar)	Papaya (without sugar)	Muskmelon (without sugar)
0	0	0	0
24	1	1	0.8
48	2	3	1
72	3	6	2
96	5	9	4
120	7	12	5
144	9	13	7
168	11	14	8

Table 3.1: Measurement of Bacterial Cellulose Thickness with Respect to Time and Sugar Concentration

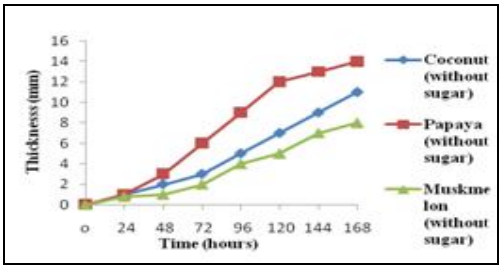

Figure 3.4: Measurement of Bacterial Cellulose Thickness with Respect to Time and Sugar Concentration

Table 3.2 and Figure 3.5 showed that, the maximum thickness of Bacterial cellulose i.e. with additional sugar was observed on the 7th day of fermentation in coconut water, papaya juice and muskmelon is 10mm, 12mm and 6mm. Hence Papaya juice medium with additional sugar gave maximum thickness when compared with other substrates.

Time (hrs)	Thickness in mm		
	Coconut (with 12% sugar)	Papaya (with 12% sugar)	Muskmelon (with 12% sugar)
0	0	0	0
24	1	1	0.5
48	2	3	1
72	4	5	2
96	6	8	3
120	7	9	5
144	8	11	6
168	10	12	6

Table 3.2: Measurement of Bacterial Cellulose Thickness with Respect to Time and Sugar Concentration

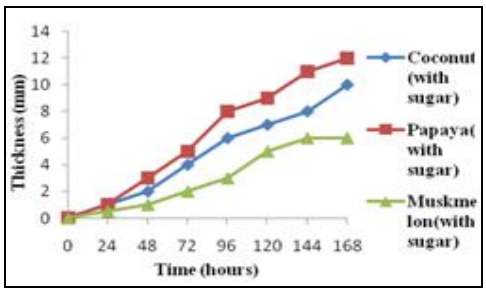

Figure 3.5: Measurement of Bacterial Cellulose Thickness with Respect to Time and Sugar Concentration

Table 3.3 and Figure 3.6 showed that the maximum amount of wet weight of BC observed for papaya juice i.e. without additional sugar was found to be 41.107g/100mL followed by Coconut water with wet weight of 27.743g/100mL and lowest wet weight of muskmelon was found to be 24.282g/100mL when compared to other substrates. Whereas the maximum amount of wet weight of

BC observed for papaya juice i.e. with additional sugar was found to be 32.813g/100mL followed by Coconut water with wet weight of 26.594g/100mL and lowest wet weight of muskmelon was found to be 16.350g/100mL when compared to other substrates. Study revealed that the papaya juice without additional sugar is more efficient for Bacterial cellulose production.

Substrates	Coconut Water	Papaya Juice	Muskmelon juice
Without sugar	27.743	41.107	24.282
With sugar	26.594	32.813	16.35

Table 3.3: Wet Weight of Bacterial Cellulose (g/100mL)

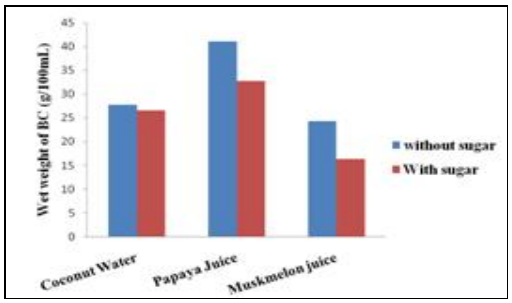

Figure 3.6: Wet Weight of Bacterial Cellulose (g/100mL)

Table 3.4 and Figure 3.7 showed that the highest reduction in weight of BC found in papaya juice i.e. without additional sugar was found to be 9.95g/100mL followed by coconut water with dry weight of 4.38g/100ml and the lowest dry weight of Bacterial cellulose was found in muskmelon juice is 2.53g/100mL. Whereas the highest reduction in weight of BC found in papaya juice i.e. with additional sugar was 6.34/100mL followed by coconut water with dry weight of 3.29g/100ml and the lowest dry weight of Bacterial cellulose was found in Muskmelon juice is 1.78g/100mL.

Substrates	Coconut water	Papaya juice	Muskmelon juice
Without sugar	4.38	9.95	2.53
With sugar	3.29	6.34	1.78

Table 3.4: Dry Weight of Bacterial Cellulose (g/100mL)

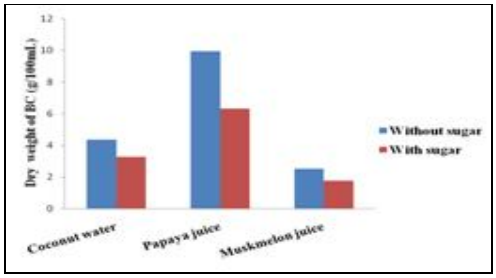

Figure 3.7: Dry Weight of Bacterial Cellulose (g/100mL)

Table 3.5 and Figure 3.8 showed that the maximum moisture content observed for coconut water, papaya juice and muskmelon juice without additional sugar was found to be 84.21%, 75.79% and 89.57% whereas the maximum moisture content observed for coconut water, papaya juice and muskmelon juice with additional sugar was found to be 87.62%, 80.67% and 89.10%.

Substrates	Coconut water	Papaya juice	Muskmelon juice
Without sugar	84.21 %	75.79 %	89.57 %
With sugar	87.62 %	80.67 %	89.10 %

Table 3.5: Moisture Content of Bacterial Cellulose

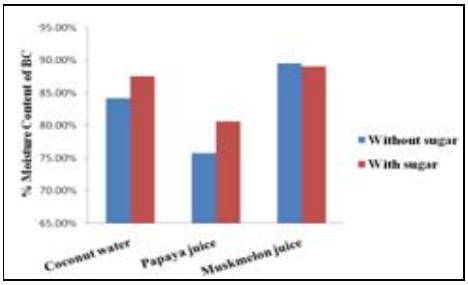

Figure 3.8: Moisture Content of Bacterial Cellulose

Table 3.6 and Figure 3.9 showed that the highest yield of Bacterial cellulose found in coconut water, papaya juice and muskmelon juice was 2.43g/100mL, 4.52 g/100mL and 1.68 g/100mL without sugar. Whereas the highest yield of Bacterial cellulose found in coconut water, papaya juice and muskmelon juice was 1.82 g/100mL, 3.51 g/100mL and 0.98 g/100mL with sugar. This reveals that papaya play significant role in Bacterial cellulose production when compared to other substrates. Figure 3.9 shows the comparison of bacterial cellulose yield from various natural carbon sources. The amount of sugar present in the natural carbon sources was estimated by DNS method. Carbohydrate analysis of Papaya juice, coconut water and muskmelon juice showed presence of 2.2, 1.8 and 1.5 % of total sugar respectively.

Substrates	Coconut water	Papaya juice	Muskmelon juice
Without sugar	2.43	4.52	1.68
With sugar	1.82	3.51	0.98

Table 3.6: Yield of Bacterial Cellulose (g/100mL)

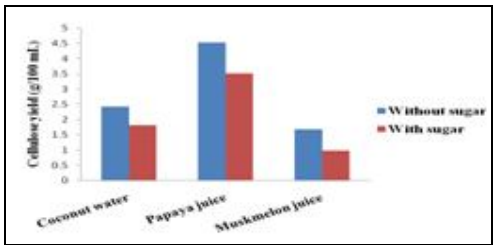

Figure 3.9: Yield of Bacterial Cellulose (g/100mL)

4. Conclusions

Due to ever increasing population in major cities, the management of waste is a major challenge for administrators and planners. Effective recycling of the waste at source is one of the most efficient waste management. Thus using this technology, coconut water generated at temples and the damaged fruits waste can be recycled at source for minimizing environmental pollution with additional economic benefit and employment generation. Bacterial cellulose was produced by *Acetobacter xylinum* using coconut water, papaya juice, muskmelon juice (wastage) as substrates where Papaya juice without additional sugar 4.52g/100 mL proved to be an efficient substrate for production of Bacterial cellulose when compared with substrates having additional sugar. In addition, it does not involve toxic and any kind of hazardous materials in producing Bacterial cellulose, which is excellent and suitable for safe environments such as medical and cosmetic applications. Cellulose production increases with use of particular carbon sources in some media, but not in others and yield is greatly affected by selection of media. It could be concluded that static condition is suitable for Bacterial cellulose production.

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