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Produce bacterial cellulose of kombucha (Khubdat Humza) from honey

Esam J. Al-Kalifawi

College of Education for Pure Science Ibn-Al-Haitham, University of Baghdad, Iraq.
Corresponding author: aesam365@yahoo.com

Abstract

Honey is a sweet food made by bees using nectar from flowers. Honey gets its sweetness from the monosaccharides fructose and glucose, and has approximately the same relative sweetness as granulated sugar and can be used as an alternative carbon source for imported sugar. Kombucha (Khubdat Humza) is composed of yeast and acetic acid bacteria especially, *Acetobacter xylinum* which forms a cellulose pellicle on sweeten tea. Kombucha (Khubdat Humza) produces bacterial cellulose pellicles, with unique purity and fine structure. It can be used in many forms, such as an emulsifier, stabilizer, dispersing agent, thickener and gelling agent. Recently, bacterial cellulose is used in many special applications such as a scaffold for tissue engineering of cartilages and bones, wound dressing, artificial skin, dental implants, vascular grafts, catheter covering dressing, and as artificial blood vessels, pharmaceutical excipient, face mask, utility in tablet formation, filter industry, papers industries, high quality speaker diaphragms, membranes, a food bulking agent, medicinal bandages, waste water treatment as well as its used in the clothing industry. Bacterial cellulose production was studied in sucrose and honey sweetened tea at 27 °C over 20 days incubation using Kombucha (Khubdat Humza). Results showed that the maximum yields of bacterial cellulose were (66.0 and 34.2 g/l) for sucrose and honey medium (100g/l and 10%) after (14^{th}-18^{th} days) fermentation period, respectively. Bacterial cellulose production using sucrose was 1.5-2 time higher than the medium containing honey during fermentation period. Temperature was essential factor on growth, where the bacterial cellulose was formed at range (20 - 50°C) and higher temperature over 50°C depressed the bacterial cellulose formation. The bacterial cellulose production increased with the increase of surface area and depth of the broth. Findings from this study suggest that the yield of bacterial cellulose from honey as alternative carbon source was not encouraging especially that honey is scarce and expensive in Iraq.

Keywords: Kombucha, Khubdat humza, Bacterial cellulose, *Acetobacter xylinum*, Honey

Introduction

Cellulose forms the basic structural foundation of the cell wall of eukaryotic plants and algae and is also found as a major constituent of the cell wall of fungi (Cannon and Anderson, 1991). It is therefore, the most abundant bio-polymer on the earth with 180 billion tons per year produced in nature (Engelhardt, 1995). A simple straight chain polymer of glucose molecules linked at the β, 1-4 position, and cellulose is the most commonly harvested from trees and cotton but is also derived from flax, jute and hemp. Bacteria also synthesize cellulose, including the genera Agrobacterium*, Rhizobium, Psuedomonas, Sarcina and Acetobacter* (Cannon and Anderson, 1991). *Acetobacter xylinum* (or reclassified *Gluconacetobacter xylinus*), a Gram-negative, rod shaped bacteria and a prodigious producer of cellulose, has been studied extensively. Initially, the extra-cellular cellulose produced by the bacteria was seen as a method for elucidating the biosynthetic pathway of cellulose, but bacterial cellulose has developed into a field of study of its own. As opposed to cotton and paper, where the purification of the cellulose product decreased the chain length, bacterial cellulose does not require remedial processing to remove unwanted polymers and contaminants (e.g. lignin, hemicellulose) and therefore, retains a greater degree of polymerization (Nishi *et al.*, 1990). This fact gives bacterial cellulose superior unidirectional strength. In a native state, bacterial cellulose also has a greater water holding capacity over a hundred times its own weight in water. These properties, along with the *in situ* ability to mold the cellulose during production, have led to innovative uses for bacterial cellulose. Included among the uses are high quality speaker diaphragms, membranes, a food bulking agent, medicinal bandages and potentially as replacement blood vessels (Serafica, 1997). To make the subject more confusing, it has been established that there are four structurally different types of cellulose, with different

properties that have been identified: cellulose I, consists of β, 1-4 glucan chains aligned in parallel, typically found in nature. Cellulose I is the cellulose which is produced in pellicle form by *G. xylinus*. In an undried state, cellulose I may be referred to as "native" cellulose (Ross *et al.*, 1991). Cellulose II, consists of anti-parallel β, 1-4 glucan chains, found in shaken culture of *G. xylinus* or after re-crystallization or industrial mercerization of cellulose I (Ross *et al.*, 1991). Cellulose III, consists of chemically treated cellulose I, cellulose IV, found in cell wall of higher plants and can be derived from chemically treating cellulose II (Haigler, and Weimer, 1991). It has been only in the last two decades that key advances have been made into the cellular activators that cause *G. xylinus* to secrete cellulose. Cellulose I (alpha) was produced predominately by bacteria and algae while cellulose I (beta) was derived from plants. However, all native cellulose contains quantities of both allomorphs (Sugiyama *et al.*, 1991). This suggested a model orientation of the cellulose chains with Iα containing a triclinic unit cell and Iβ containing a monoclinic unit cell. Furthermore, silver labeling proved that all the reducing groups were orientated the same way (Hieta *et al.*, 1984). Bacterial Cellulose has gained attention in the research realm for the favorable properties it possesses; such as its remarkable mechanical properties in both dry and wet states, porosity, water absorbency, moldability, biodegrability and excellent biological affinity (Shoda, and Sugano, 2005). Because of these properties, BC has a wide range of potential applications including use as a separation medium for water treatment (Choi *et al.*, 2004), a specialty carrier for battery fluids and fuel cells (Brown, 1990), a mixing agent, a viscosity modifier (Jonas and Farah, 1998), immobilization matrices of proteins or chromatography substances (Sokolnicki *et al.*, 2006) etc. The prevalent application of BC is in the biomedical field, as it is highly useful for wound dressing (Hamlyn *et al.*, 1997; Cienchanska, 2004; Legeza *et al.*, 2004;), artificial skin (Jonas and Farah, 1998; Czaja *et al.*, 2007), dental implants; vascular grafts; catheter covering dressing (Wan and Millon, 2005) and as artificial blood vessels (Klemm *et al.*, 2001; Backdahl *et al.*, 2006; Wan *et al.*, 2006). Honey is a mixture of sugars and other compounds. With respect to carbohydrates, honey is mainly fructose (about 38.5%) and glucose (about 31.0%), making it similar to the synthetically produced inverted sugar syrup, which is approximately 48% fructose, 47% glucose, and 5% sucrose. Honey's remaining carbohydrates include maltose, sucrose, and other complex carbohydrates (Mandal and Mandal, 2011). As with all nutritive sweeteners, honey is mostly sugars and contains only trace amounts of vitamins or minerals (Sherlock *et al.*, 2010; French *et al.*, 2005). Honey also contains tiny amounts of several compounds thought to function as antioxidants, including chrysin, pinobanksin, vitamin C, catalase, and pinocembrin (Martos *et al.*, 2000; Gheldof *et al.*, 2002). The specific composition of any batch of honey depends on the flowers available to the bees that produced the honey (Sherlock *et al.*, 2010). The present study aims to exploit the production of bacterial cellulose using the honey as a source of carbon substitute for sugar imported from abroad.

Materials and Methods

Microorganism: Starter culture of kombucha (Khubdat Humza) was of Iranian origin and was provided by Iraqi citizen. The tea sample was activated every 2 weeks by the procedure described by (Chen and Liu, 2000).

Culture media and cultivation: Substrate for kombucha fermentation was prepared by adding 100g/l of commercial sucrose to tap water and after boiling 10g/l of dry black tea was added. The tea leaves were steeped for 15minutes and removed by filtration, after cooling to about 30°C and it incubated under aerobic conditions at 28°C.

Effect of various concentrations of honey and sucrose on the bacterial cellulose formation

A. Preparation of honey solutions: Different amounts of honey with concentration (82.12%) as carbon source were tested on wet bacterial cellulose pellicle formation in g/l, these amounts were 12, 24, 30, 60, 121, 182 and 243ml/l.

B. Effect of various concentrations of sucrose on the bacterial cellulose formation: Different amounts of sugar as carbon source were tested on wet bacterial cellulose pellicle formation in g/l, these amounts were 70, 80, 90, 100, 130, 160 and 190g/l. The bacterial cellulose produced in different concentrations of date syrup or sucrose was weighed according to the following equation:

• weight of bacterial cellulose (g/l) = total weight of beaker containing cellulose + tea broth – weight of beaker only + tea broth

• the yield of the cellulose:- yield % = wet weight of bacterial cellulose (g/l) ÷ honey or sucrose concentration (g/l), described by (Akhter *et al.*, 2013).

Effect of different temperature on bacterial cellulose production: Tea broth was prepared by adding %10 honey and 10g/l black tea and incubated at different temperature (20°C, 30°C, 40°C, 50°C, 60°C, 70°C and 80°C) and tested their effect on bacterial cellulose formation.

Effect of prolong fermentation on bacterial cellulose production: Tea broth was prepared by adding %10 honey and 10g/l black tea and

incubated at different periods (3days , 6days, 9days, 12days, 15days, 18days and 21days) and tested their effect on bacterial cellulose formation.

Effect of surface area and depth of culture medium on bacterial cellulose production: Tea broth with %10 honey and 10g/l black tea was prepared in different container's size. The bacterial cellulose produced was weighed after 14 days of the fermentation. Then, the effect of surface area and depth of the culture medium on cellulose production was examined.

Results

The bacterial cellulose depends on the supply of a carbon source, it cannot produce the cellulose. In Table (1), the experimental results conducted that the concentration of sucrose and honey at (100g/l and 10%) produced the highest yield of pellicle (66.0 and 34.2g/l) and increasing sucrose and honey concentration from (130-190g/l) and (15-20%) respectively produced a gradual decrease in the yield.

Bacterial cellulose production was strongly affected by the incubation temperature. A gradual drop occurred in fermentation tea broth with high temperature (60-80°C) where the bacterial pellicle was not formed and tea broth was dark brown in colour, odourless and clear (Table 2).

Table (3) shows the changes in wet weight of the bacterial cellulose pellicle as the fermentation progressed, and the yield of bacterial cellulose as the fermentation proceeded for 21 days. Both the wet weight and yield of bacterial cellulose increased with fermentation time. The bacterial cellulose yield increased progressively over the whole course of fermentation, with a maximum production of 42.20% with a wet weight of bacterial cellulose of 34.66g/l after 18^{th} days.

Table (4) shows the amount of bacterial cellulose produced in cultures with different volumes and surface areas, the bacterial cellulose production increased with an increase of surface area. The round container with the greatest surface area ($227cm^2$), depth (6.6cm) produced highest bacterial cellulose (98.12g/l) while, other container has surface area ($28.26cm^2$), depth (40.2cm) produce bacterial cellulose (18.20g/l).

Table (1): Effect of different sucrose and honey concentrations on the yield of bacterial cellulose.

Sucrose conc. g/l	honey conc. %	Wet weight of bacterial cellulose (g/l)		Yield %	
		Sucrose	Honey	Sucrose	Honey
70	1	30.11	18.24	43.01	22.21
80	2	35.22	23.44	44.02	28.54
90	2.5	45,00	27.00	50.00	32.87
100	5	66.00	30.11	66.00	36.66
130	10	32.23	34.20	24.79	41.64
160	15	26.66	29.55	16.66	35.98
190	20	21.22	20.88	11.16	25.42

Table (2): effect of different temperature on the bacterial cellulose of kombucha tea.

Temperature (°C)	Colour	Clearity	Pellicle formation	Odour
20	Normal	Turbid	Formed	+ve
30	Normal	Turbid	Formed	+ve
40	Normal	Turbid	Formed	+ve
50	Normal	Turbid	Formed	+ve
60	Normal	Clear	Nill	-ve
70	Dark	Clear	Nill	-ve
80	Dark	Clear	Nill	-ve

Table (3): Effect of Incubation periods on the yield of bacterial cellulose.

Incubation period Days	Wet weight of bacterial cellulose (g/l)	Yield %
3	19.22	23.40
6	20.33	24.75
9	23.00	28.00
12	30.12	36.67
15	33.00	40.18
18	34.66	42.20
21	30.50	37.14

Table (4): Effect of culture surface area & depth of containers on the bacterial cellulose.

No. of container	Depth (cm)	Surface area (cm^2)	Surface area depth(cm)	Wet weight of pellicle (g/l)
1	40.2	28.26	0.70	18.20
2	34.2	29.20	0.90	20.00
3	8.4	78.50	7.85	33.11
4	6.4	78.50	12.30	40.25
5	5.4	130.50	24.20	50.95
6	6.8	176.63	25.97	77.43
7	6.6	227.00	34.40	98.12

Discussion

Kombucha is a popular beverage among traditional fermented foods across the world. It is symbiotic relationship between acetic acid bacteria and yeasts in a sugar tea. Cellulose produced during the fermentation by Acetic acid bacteria especially, *A-Xylinum* appears as thick film on the top of tea broth which enhances the association formed between bacteria and yeasts (Choi *et al*., 2004; Czaja *et al.*, 2007). Tea used as nitrogen source, chemical composition of tea leaves has been thoroughly studied. The main constituents of black tea, the oxidation of polyphenols during processing leads to the formation of catechins and gallic acid complexes such as thea¯avins, thea¯avinic acids, thear ubigins or theasinensis, and of proanthocyanidin polymers (Akhter *et al*., 2013; Malbaša *et al.*, 2006). Methylxantines are present with 2±4% as caffeine and a small amount of theophylline and of theobromine (Dashti and Morshedi, 2000). Tea contains many amino acids, but theanine, specific to the tea plant, is the most abundant, accounting for 50% of the total amino acids, chlorophyll, carotenoids, lipids, and volatile compounds. Tea also contains carbohydrates, vitamins E, K, A, low levels of B vitamins and vitamin C. All these material involved in kombucha growing (Wagner *et al.*, 2013). Almost all the living microorganisms require carbon source for their general growth and metabolism. Also, carbon is a component of all the substances that constitute protoplasm (Caldwell, 2000). According to (Frank, 1995), the 'mother' (starter) culture of bacterial cellulose depends on the supply of a carbon source (sugar, mainly sucrose) as it cannot produce the cellulose in adequate quantities on its own. Previously, effects of sugars such as sucrose, lactose, glucose and fructose at various concentrations (50– 150 g/l) on the metabolism of the tea fungus and on the formation of ethanol and lactic acid have been studied by (Reiss, 1994). In the present study, results of our preliminary experiments conducted revealed that the concentration of sucrose and honey present in the tea broth affects the synthesis of bacterial cellulose Table (1), and these results are similar to the previous report by (Masaoka *et al.*, 1993). Sucrose at a concentration of 100 g/l produced the highest yield of cellulose, honey at a concentration 10% produced the highest yield of cellulose and increasing the sucrose and honey concentration more than (100g/l and 10%) produced a gradual decrease in the yield. This finding agrees with an earlier report published by (Embuscado *et al.*, 1994), where sugar utilization resulted in a decrease in cellulose production as sugar concentration increased. Therefore, an appropriate level of sugar is necessary for optimum bacterial cellulose production. Based on these preliminary findings Table (1), of more metabolic products during the course of fermentation when a substantial amount of sugar is present in the tea broth might explain this result wherein more metabolic products would lead to product inhibition (Dashti and Morshedi, 2000). Another possible explanation is unequal rates of transport of critical cell materials (nutrients) and rates of the nutrients' utilization. According to (Chen and Liu, 2000), the rate of removal of potentially harmful substances must balance the production of bacterial cellulose. Therefore, the greater the amount of sugar in the tea broth, the stronger the hindrance to bacterial cellulose synthesis would be. Bacterial cellulose production using sucrose was 1.5-2 time higher than the medium containing honey during fermentation period. Our interpretation to this topic belong to honey has its own bacterial profile and may disrupt the balance of yeast and bacteria in the scoby. Additionally, honey may include organic material that might disturb the scoby or attract mold. This finding agree with (Embuscado *et al.*, 1994).

Temperature effects on the cellulose pellicle production , the typical temperature to growing kombucha colony is between 20-50°C, in this range of temperature we harvest the biggest wet weight and yield of the bacterial cellulose Table (2), where the high temperature prevents the growth of bacterial cellulose and pellicle formation and this is similar to (Jayabalan *et al.*, 2008).

To study the productivity of bacterial cellulose during prolonged fermentation, the bacterial cellulose yield produced by tea broth with honey at 10% was measured over a 21 day fermentation period Table (3). The changes in wet weight of the bacterial cellulose pellicle shows as the fermentation progressed, and the yield of bacterial cellulose as the fermentation proceeded for 21 days. Both the wet weight and yield of bacterial cellulose increased with fermentation time. The bacterial cellulose yield increased progressively over the whole course of fermentation, with a maximum production of after 18 days , production remained constant thereafter. According to (Frank, 1995), this pattern occurred because by day 17–18 of fermentation, the reserved glucose was almost exhausted and the metabolites had reached maximum production. Therefore, an increase in the bacterial cellulose yield of only 2.5% was observed. Similar profiles have been observed in static fermentation experiments conducted by other researchers (Masaoka *et al.*, 1993; Borzani and Desouza, 1995). They reported that the wet weight and the yield of bacterial cellulose increased sharply after a few days of induction until the rate reached a maximum after 2 weeks. The mechanism of bacterial cellulose formation has been described in detail by (Malbaša *et al.*, 2006; Legeza *et al.*, 2004). In the initial stage, the bacteria increase their population by using dissolved oxygen and produce a certain amount of cellulose in the liquid phase, as observed by the appearance of turbidity. When the dissolved oxygen is depleted, bacteria existing only in the vicinity of the surface area can maintain their activity to produce bacterial cellulose. Although the bacteria may undergo rapid cell division, the population on the surface region does not increase exponentially, but should reach a certain equilibrium number, as most of them are occluded in the bacterial cellulose pellicle and brought into depth. Those bacteria below the surface are not dead but asleep, so that they can be reactivated and used as the seed for a new culture (Yamanaka and Watanabe, 1994). In a static culture, the bacterial cellulose pellicle is formed at the air-liquid interface as the aerobic bacteria produce cellulose only in the vicinity of the surface. Hence, the presence study was conducted to investigate the influence of the surface area and depth of the culture medium on pellicle formation (Iguchi *et al.*, 2000). According to (Frank, 1995), when the bacterial cellulose culture floats on the surface, it first grows outwards until the surface of the solution is fully covered, and followed by this, it grows thicker. Hence, the upper-most layer is always considered to be the newest. Table (4) shows the amount of bacterial cellulose produced in cultures with different volumes and surface areas and accordingly, bacterial cellulose production increased with an increase of surface area. As the metabolic processes of tea fungus depend on fresh air it is very important that care is taken to ensure a sufficient supply of oxygen. The results of the present study shows surface area played a more significant role in the formation of bacterial cellulose than did the volume of the culture medium. Malbaša *et al.* (2006) also proposed that the culture volume does not influence bacterial cellulose production; they also reported that a continuous bacterial cellulose layer fails to form in a vessel with a tapered wall, such as a conical flask. The effect of depth of the culture medium on bacterial cellulose production was examined by fermenting the tea fungus in containers with different volumes and depths. When the depth of the culture medium was not greatly different, the production of bacterial cellulose depended mainly on the volume of the medium. At these depths, cultures with a larger volume of culture medium produced more bacterial cellulose. These data agree with those reported by (Okiyama *et al.,* 1992), who found that a deep column container only generated a small amount of bacterial cellulose. These results can be explained as follows: cells produce carbon dioxide, which is trapped in the pellicle (Schramm and Hestrin, 1954), and the deeper the culture medium the more carbon dioxide accumulates in the pellicle. When the inside of the pellicle is less aerobic, cell growth and pellicle formation are inhibited because acetic acid bacteria are strict aerobes. To conclude, surface area (ratio of surface area to depth) of the culture medium played an important role in bacterial cellulose formation. Therefore, to enhance bacterial cellulose production, the culture medium used for fermentation should be shallow and should occur in a container with a very wide opening.

Conclusion

Findings from this study we are concluded that the yield of bacterial cellulose from honey as alternative carbon source was not encouraging especially that honey is scarce and expensive in Iraq.

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