Factors Influence on the yield of Bacterial Cellulose of Kombucha (Khubdat Humza)

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Received 20, December, 2013
Accepted 5, February, 2013

Abstract:

Kombucha(Khubdat Humza) is composed of yeast and acetic acid bacteria especially, *Acetobacter xylinum* which forms a cellulose pellicle on tea broth. 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 but these are generally subsidiary to its most important use of holding on to water. Recently, bacterial cellulose is used in many special applications such as a scaffold for tissue engineering of cartilages and blood vessels, also for artificial skin for temporary covering of wounds, as well as its used in the clothing industry. The yield of cellulose produced were investigated in this study, the tea broth was fermented naturally over a period of up to 20 days in the presence of different amounts of black tea and sucrose as nitrogen and carbon sources. 10g/L black tea produced highest weight of bacterial cellulose (55.46g/L) and 100g/L sucrose also exhibited high amount of pellicle (63.58g/L). Temperature was essential factor on growth, where the pellicle was formed at range (20°C - 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 cellulose depends on many factors that need to be optimized to achieve maximum yield.

Key Words: Kombucha (Khubdat Humza), Bacterial Cellulose, *Acetobacter xylinum*

Introduction:

Kombucha is one of the highly popular fermented traditional beverages consumed for potential health benefits. Consumption of Kombucha is reported to cure some of the common ailments like arthritis, indigestion, various types of cancer, hepatotoxicity, etc. [1, 2, 3]. Kombucha fermentation is brought about by the symbiosis of yeast species and acetic acid bacteria [4] and the harboring microbes or the starter culture has been declared to be safe by the US Food and Drug Administration [5], which further paves the way for exploring from consumer's safety prospects. The microbial composition has well studied, the main acetic acid bacteria include *A. acetic, A. xylinum* and others [6,7], yeasts break sucrose into glucose and fructose [8], glucose subunit that form the cellulose microfibril are excreted through pores in the cell wall of the acetic acid bacteria as *A. xylinum* [9,10,11,12].

Cellulose is the most abundant bio-polymer and has been widely accepted as the excipient in pharmaceutical and industries. It has found its utility in tablet formation [13]. The molecular formula of bacterial cellulose $(C_6H_{10}O_5)_n$ is the same as that of plant cellulose, but their physical and chemical are different [14,15].

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Cellulose is polysaccharide which forms an integral part of most plant materials. However, in several instances, commercially available plant based cellulose are impure due to the presence of high amounts of lignin or hemi-cellulose. Compared to the plant cellulose, bacterial cellulose can be synthesized (extracellularly) by bacteria such as *Acetobactor xylinum*. Bacterial cellulose is reported to possess several advantages which include: high purity, better mechanical strength, crystallinity and hydrophilicity with same chemical structure as that of Plant cellulose [16,14]. Considering these facts, it is highly essential and a pre-requisite to characterize the microbial cellulose produced from Kombucha for commercial use and applications.

Presently bacterial cellulose is receiving great attention and being widely investigated as a new type of scaffold material due to it fine fiber net work, biocompatibility, high water holding capacity, high tensile strength [17], high crystalline , high degree of polymerization, high purity , elasticity, durability, non -toxic and non-allergic [18,19,20,21,22,23,24].

In food applications the Bacterial cellulose was used as an additive, emulsifier, dietary fiber, edible preservative and as a barrier against bacterial growth [25, 24]. Recently, bacterial cellulose is used in many special applications such as a scaffold for tissue engineering of cartilages and blood vessels [26, 27, 28], as well as for artificial skin for temporary covering of wounds [29]. Purified and dried bacterial cellulose was converted to a membrane to be used in the separation processes such as ultrafilteration, gas permeation and vapor permeation, and used in paper manufacture [30, 31]. That culture conditions such as type of strain, temperature of growth, carbon source, pH and the method of gel purification weather it is done by chemical agent, concentration, temperature or exposition time possibly affected the physical properties of the resultant membranes.

The main aim of the present study was to investigate the microbial cellulose and affected it by some factor as nitrogen and carbon sources, temperature, incubation period, and surface area, depth during fermentation of a natural broth.

Materials and Methods:

Microorganism

Starter culture of Kombucha (Khubdat Humza) was of Turkish origin and was provided by Iraqi citizen. The tea sample was activated every 2 weeks by the procedure described by [32].

Culture media and cultivation

Substrate for kombucha fermentation was prepared by adding 70g/L of commercial sucrose to

tap water and after boiling 5g/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 different concentrations of black tea on the bacterial cellulose production

Sweetened tea broth was prepared by different concentrations of black tea 5, 10, 15, 30, 60, 90 and 120 g/L. these concentrations of tea were chosen on the wet weight of cellulose produced in g/L.

Effect of various concentrations of sucrose on the bacterial cellulose formation

Different amounts of sucrose 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 tea 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) ÷ tea or sucrose concentration (g/L), described by [33].

Effect of different temperature on bacterial cellulose production

Tea broth was prepared by adding 100g/L sucrose 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 100g/L sucrose 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 100g/L sucrose 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:

Black tea used to have the greatest bacterial cellulose. At concentration of 5g/L, the inhibitory affect was observe of but 10g/L black tea was exhibited a maximum value of bacterial pellicle compared with another set of seven tea concentrations, where the weight of bacterial pellicle decrease with the increase of tea concentrations as in table (1).

Table 1. Effect of different tea concentrations on the yield of bacterial cellulose

Tea concentration (g/L)	Wet weight of bacterial cellulose (g/L)	Yield %
5	23.40	468.0
10	55.46	554.6
15	28.00	186.6
30	25.85	86.16
60	24.60	41.00
90	23.66	26.28
120	23.00	19.16

Also, the bacterial cellulose depends on the supply of a carbon source, it cannot produce the cellulose. In table (2), the experimental results conducted that the concentration of sucrose at 100g/L produced the highest yield of pellicle and increasing sucrose concentration from (130g/L - 190g/L) produced a gradual decrease in the yield.

Table 2. Effect of different sugar concentrations on the yield of bacterial cellulose

Sucrose concentration (g/L)	Wet weight of bacterial cellulose (g/L)	Yield %
70	31.50	45.00
80	38.00	47.50
90	52.00	57.77
100	63.58	63.58
130	29.45	22.65
160	27.00	16.88
190	24.95	13.13

Bacterial cellulose production was strongly affected by the incubation temperature. A gradual drop occurred in fermentation tea broth with high temperature (60°C - 80°C) where the bacterial pellicle was not formed and tea broth was dark brown in colour, odourless and clear table (3).

Table 3. 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 (4) 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 63.58g/l with a wet weight of bacterial cellulose of 63.58g/l after 18 days.

Table 4. Effect of Incubation periods on the yield of bacterial cellulose

Incubation period Days	Wet weight of bacterial cellulose (g/L)	Yield %
3	18.00	18.00
6	24.95	24.95
9	29.45	29.45
12	31.50	31.50
15	48.88	48.88
18	63.58	63.58
21	50.11	50.11

Table (5) 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 (227cm2), depth (6.6cm) produced highest bacterial cellulose (104.80g/L) while, other container has surface area (28.26cm2), depth (40.2cm) produce bacterial cellulose (20.5g/L).

Table 5. Effect of culture surface area & depth of containers on the bacterial cellulose

No. of container	Depth (cm)	Surface area (cm2)	Surface area depth(cm)	Wet weight of pellicle (g/L)
1	40.2	28.26	0.70	20.50
2	34.2	29.20	0.90	21.30
3	8.4	78.50	7.85	39.80
4	6.4	78.50	12.30	41.00
5	5.4	130.50	24.20	66.90
6	6.8	176.63	25.97	85.30
7	6.6	227.00	34.40	104.80

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 [4,7]. Tea used as nitrogen source in different concentrations, Table (1) shows the highest yield of bacterial cellulose is obtained at 10g/L tea, as [33,34] discussed the tea prepared with 10g/L (1%) tea w/v give the highest yield of microbial cellulose, whereas [35] found the tea concentration of 1.5g/l give highest yield of microbial cellulose. 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 [36]. According to [37], 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

[38]. In the present study, results of our preliminary experiments conducted revealed that the concentration of sucrose present in the tea broth affects the synthesis of bacterial cellulose Table (2), and these results are similar to the previous report by [39]. Sucrose at a concentration of 100 g/l produced the highest yield of cellulose, and increasing the sucrose concentration more than 100 g/l produced a gradual decrease in the yield. This finding agrees with an earlier report published by [40], 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 (2). 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 [37]. Another possible explanation is unequal rates of transport of critical cell materials (nutrients) and rates of the nutrients' utilization. According to [36], the rate of removal of potentially harmful substances must balance the production of bacterial cellulose. Therefore, the greater the amount of sucrose in the tea broth, the stronger the hindrance to bacterial cellulose synthesis would be. 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 (3), where the high temperature prevents the growth of bacterial cellulose and pellicle formation and this is similar to [41].

To study the productivity of bacterial cellulose during prolonged fermentation, the bacterial cellulose yield produced by tea broth with sucrose at 100 g/l was
measured over a 21 day fermentation period. 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 [37], 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 (39,42). 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 [26,14]. 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 [43]. 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 [44]. According to [37], 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 (5) 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. [39] 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 [45], 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 [46], 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: 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:

Results of the present study demonstrated bacterial cellulose to be produced during static fermentation of black tea broth. over a period of 8days. The concentration of sucrose, which was used as the main carbon source, the concentration of black tea, which was used as the main nitrogen source, the temperatures and the periods of incubation, also the depth and the surface area highly influenced the yield of bacterial cellulose.

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العوامل المؤثرة على انتاج السليلوز الجرثومي للكمبوشا (كبده حمزة)

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الخلاصة:

الكمبوشا (كبده حمزة) تتكون من الخمائر وجراثيم حامض الخليك وخصوصا جرثومة *Acetobacter xylinum* والتي تكون غشاء سليلوزي على سائل الشاي. أن مزرعة الكمبوشا تنتج السليلوز الجرثومي بنطاق واسع مع نقاء فريد، ويمكن استخدامه في أشكال كثيرة، مثل مستحلب، مثبت، عامل تفريق، مثخن وكيل التبلور ولكن هذه عادة ما تكون تابعة لاستخدامه الأهم كمحتفظ للمياه. حاليا يستخدم السليلوز الجرثومي في العديد من التطبيقات مثل هيكل لهندسة الانسجة للغضاريف والأوعية الدموية كذلك كجلد صناعي للتغطية المؤقتة للجروح، فضلا عن استخدامه في صناعة الملابس .في هذه الدراسة بحث انتاج السليلوز، حيث خمر سائل الشاي طبيعيا لفترة اكثر من 20 يوما بوجود كميات مختلفة من الشاي الاسود والسكر كمصادر للنتروجين والكربون. ان 10 غم من الشاي الاسود انتج اعلى وزن من السليلوز الجرثومي (55.46 غم /لتر)، كذلك 100 غم /لتر سكر اظهر انتاج كميات كبيرة من الغشاء السليلوزي (63.58 غم /لتر). ان درجة الحرارة عامل اساسي مؤثر على النمو، حيث لوحظ ان الغشاء السليلوزي يتكون عند معدل 20-50 °م وان درجة الحرارة اكثر من 50 °م تقلل من انتاج السليلوز. انتاج السليلوز الجرثومي يزداد مع زيادة المساحة السطحية والعمق للسائل. تشير نتائج الدراسة الحالية الى ان انتاج السليلوز يعتمد على عدة عوامل اساسية من اجل الحصول على اعلى انتاج.