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PRODUCTION OF MICROBIAL CELLULOSE BY TEA FUNGUS "KOMBUCHA"

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ABSTRACT

In seeking economic production of microbial cellulose, the available and low cost commercial kombucha starter, contains mainly *Acetobacter xylinum* and *Saccharomyces sp.*, were used under static condition. The best medium composition was black tea extract (1.0 g /100 ml H_2O), sucrose (20%) and corn steep liquor, CSL (1%) at 26-28ºC for 10 days. The cellulose yield (1.3 g /100 ml) and productive rate (1.3 X 10^{-3} g / day /ml) were higher than some reported values. Addition of folic acid or its building block *p*-aminobenzoic acid at additive concentrations 0.20% led to doubling the yield (2.37 and 2.43 g / 100 ml culture respectively) and the productive rate (2.37 X 10^{-3} and 2.43 X 10^{-3} g / day / ml respectively). Scanning electron micrograph showed the structure of the produced microbial cellulose fibrils without any microbial flora after treatment with 1% NaOH.

INTRODUCTION

Cellulose is considered the most abundant biopolymer in plant kingdom. It forms the main structure of the cell wall in most plants and algae; in addition to constituting most of the cotton and > 50% of the wood structures. Both microbial and plant cellulose consist of β-1,4-glucose units but the former contains 2000-6000 monomer while the later contains 13000- 14000 glucose unit (**Jonas and Farah 1998**). Cellulose is used in industry for the production of high strength and biodegradable materials e.g. paper, food additives and artificial blood vessels and skin (**Jeong and Lee 2000 and Klemm *et al* 2001**). Microbial cellulose has the advantage over plant cellulose because of its high purity, light weight, fine network structure and fast biodegradation in addition to its exceptional mechanical strength especially in wet state, enormous water retention value, low roughness of the inner surface and high hydrophilicity which makes the microbial cellulose suitable for medical applications (**Klemm *et al* 2001**). However, its disadvantages are its expenses and low yields (**Tsuchida and Yoshinaga 1997**). Therefore, identifying the microorganisms and the suitable conditions that minimize the cost and maximize the yields of microbial cellulose production is of great concern.

Several organisms have been tested for cellulose production e.g. *Acetobacter, Aerobacter, Achromobacter, Agrobacterium, Pseudomonas, Rhizobium* and *Sarcina* where the produced cellulose could be in the form of fibrils, pellicle, ribbons, crystalline or amorphous cellulose (**Toyosaki *et al* 1995; Jonas and Farah 1998 and Astley *et al* 2001**). The chemical composition of the fermentation media and the use of some additives were found to affect cellulose yield. The use of glucose-fructose (**Yang *et al* 1998**) or molasses (**Bae and Shoda 2005**) as a carbon source, and corn steep liquor (CSL) as a nitrogen source (**Noro *et al* 2004**) increased the yield. Some vitamins and natural products acted as stimulators for cellulose production e.g. pyridoxine, nicotinic acid, *p*-aminobenzoic acid, biotin, ethanol, caffeine and xanthines (**Fontana *et al* 1991; Jonas and Farah 1998 and Park *et al* 2003**). Kombucha is a symbiosis of bacteria (*Acetobacter xylinum, Acetobacter xylinoides* and *Bacterium gluconium*) and some yeast species e.g. *Saccharomyces cerevisiae, Schizosccharomyces pombe* and *Saccharomy-*

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codes ludwigii (**Sreeramulu *et al* 2000**) that is available commercially at a low cost. It is cultured in sugared black tea where the fermentation media contain small amounts of alcohol, acetic acid, gluconic acid, lactic acid and some antibiotics (**Mayser *et al* 1995**). The present work examines the production of microbial cellulose by Kombucha and seeks the suitable low cost condition.

MATERIALS AND METHODS

Kombucha starter was obtained from Günther W. Frank, Genossenschaftsstr, Birkenfeld, Germany. Corn steep liquor (CSL) containing 3-4% nitrogen was obtained from Egyptian Starch & Glucose Company, Helwan, Kotseca. Chemicals were reagent grade and obtained from Sigma or Aldrich Chemical Companies. Micrographs of dried cellulose were recorded by using Scanning Electron Microscope, JEOL T 330A, Central Laboratory, Faculty of Agriculture, Ain Shams University.

The commercial Kombucha starter was cultivated in a tea broth prepared by extracting 0.5 g black tea by 100 ml boiled water; the fermentation culture contained 10% sucrose and renewed each 12 days (**Sreeramulu *et al* 2000**). The effect of fermentation period (up to 12 days) on cellulose yields was studied; measuring pH was also performed each 48 hour. The tested sucrose concentrations were 10-45% while tea extracts were 0.5-4.0 g/100ml water; CSL concentrations were 0-9%. The best condition obtained from each experiment was adopted in the following experiments. Additives, *p*-aminobenzoic acid, folic acid, lactic acid, nicotinic acid and glycerol, were tested in various concentrations. Additives were examined in tea broth culture (1.0g / 100 ml) containing Kombucha microorganisms, 20% sucrose and 1% CSL for 10 days. Additive concentrations were 0.1, 0.2 and 0.3%, except for glycerol concentrations which were 1.0, 2.0 and 3.0%. All cultures were incubated statically at 26-28ºC. At the end of the fermentation period, cellulose layer was removed and washed twice with distilled water then treated with NaOH (1%) at 90ºC for 30 minutes (**Yang *et al* 1998**). The produced cellulose layer was washed with distilled water and dried at 50ºC till constant weight was reached.

RESULTS AND DISCUSSION

Commercial Kombucha starter was used for cellulose production as a cheap and available source of microorganisms cultured in a low cost medium, tea broth under static condition. Kombucha contains mainly two organisms, *Acetobacter xylinum* and *Saccharomyces sp. Acetobacter xylinum* was found to produce cellulose under various conditions (**Yamanaka 1989; Tsuchida and Yoshinaga 1997 and Krystynowicz *et al* 2005**) while *Saccharomyces* extracts stimulated its production (**Yang *et al* 1998**). The produced cellulose was treated with 1% NaOH to get rid of the cell mass then dried at 50ºC to constant weight; using higher temperature, 80ºC (**Yang *et al* 1998**), yielded darker cellulose. Cellulose yield and pH were monitored at various intervals up to 12 days. Cellulose yield increased gradually with time; however, the increasing rate of yield decreased about 6 folds after 6 days, where the slopes before and after 6 days were 0.057 (R = 0.995) and 0.010 (R = 0.976) respectively, which could be attributed to the encountered lowering in the pH as shown in **Fig. (1)**. Most reports indicated an optimal pH range for cellulose production 4-7 (**Jonas and Farah 1998**). The drop in the pH of the fermented brewer during the incubation period resulted from the conversion of glucose to gluconic acid (**Yang *et al* 1998**) and the production of other organic acids.

With respect to the optimum sucrose concentration for cellulose production, it was found to be 20% in the culture broth medium, where more sucrose concentration led to sharp decline in cellulose production (slope = - 0.017, R = 0.943) as shown in **Fig. (2)**. It was reported that increasing sucrose concentration led to increasing the cell yield (**Yang *et al* 1998**), which could be at the expenses of the cellulose production. Results presented in **Fig. (3)** indicated that the best tea extract for cellulose production ranged from 0.5 to 1.0 g / 100 ml; thereafter, a sharp decrease with a slope – 0.268 (R = 0.954) was recorded. Corn steep liquor (CSL) was found to be effective as a nitrogen source for cellulose production. A maximum cellulose yield (0.661%) was obtained by *Acetobacter xylinum* in a medium containing 6% CSL (**Yang *et al* 1998**). However, in the present investigation, a maximum cellulose yield (1.30%) was obtained at 1.0% CSL. CSL stimulated both cell growth and cellulose production in a *A. xylinum* culture (**Tsuchida and Yoshinaga 1997**), which could account for the observed drop in cellulose production with increasing the CSL percentage **(Fig. 4)**.

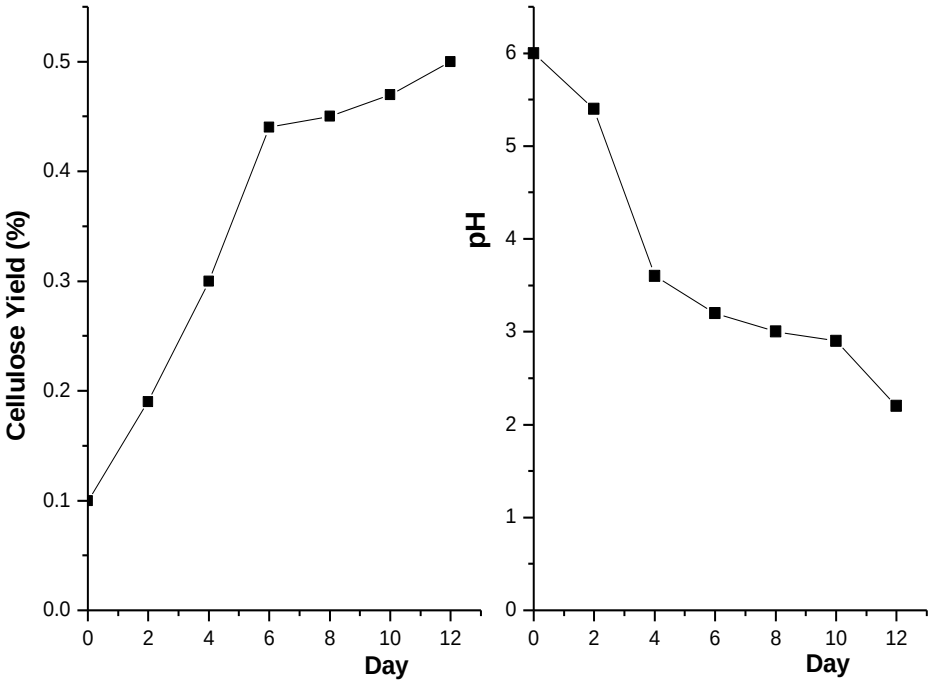


Fig.1. Effect of fermentation period on cellulose yield and pH using tea extract (0.5 g/100 ml) media containing 10% sucrose

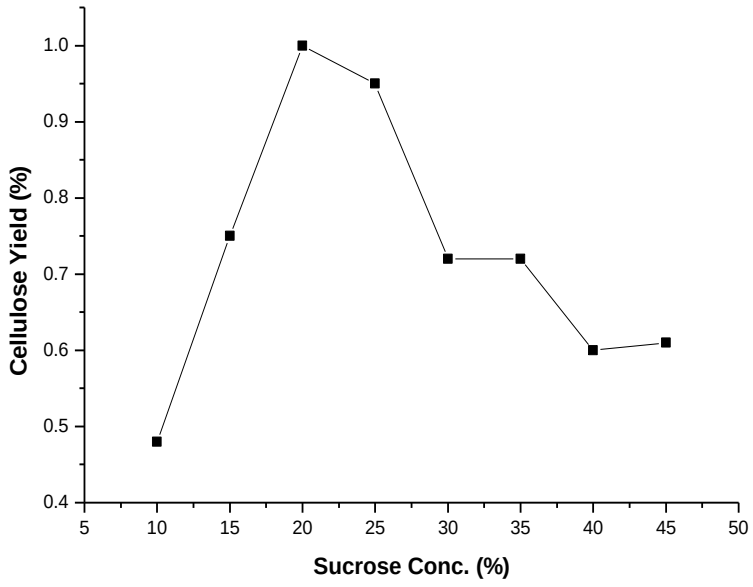


Fig. 2. Effect of sucrose concentration on cellulose production using tea extract (0.5 g/100 ml) media for 10 days

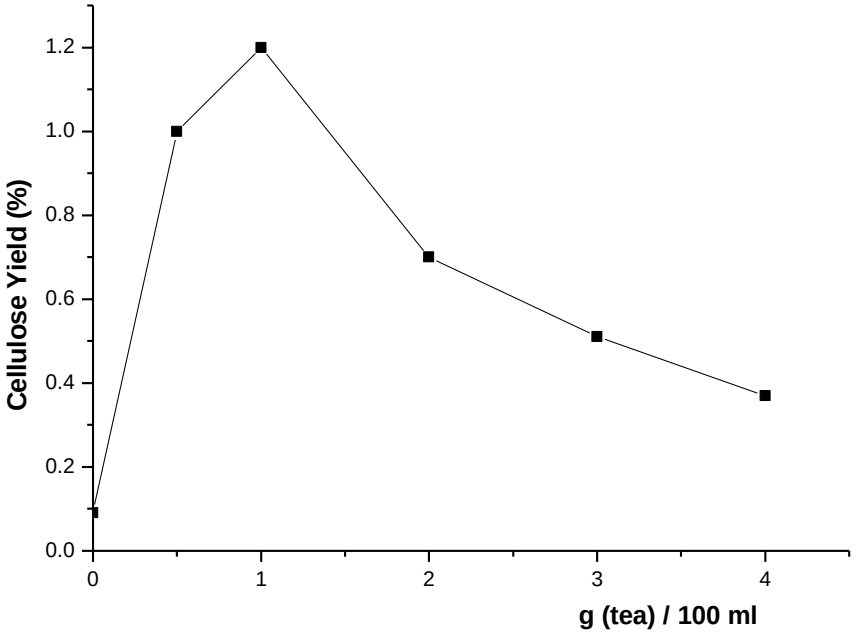


Fig. 3. Effect of tea extract concentration on cellulose production using 20% sucrose for 10 days

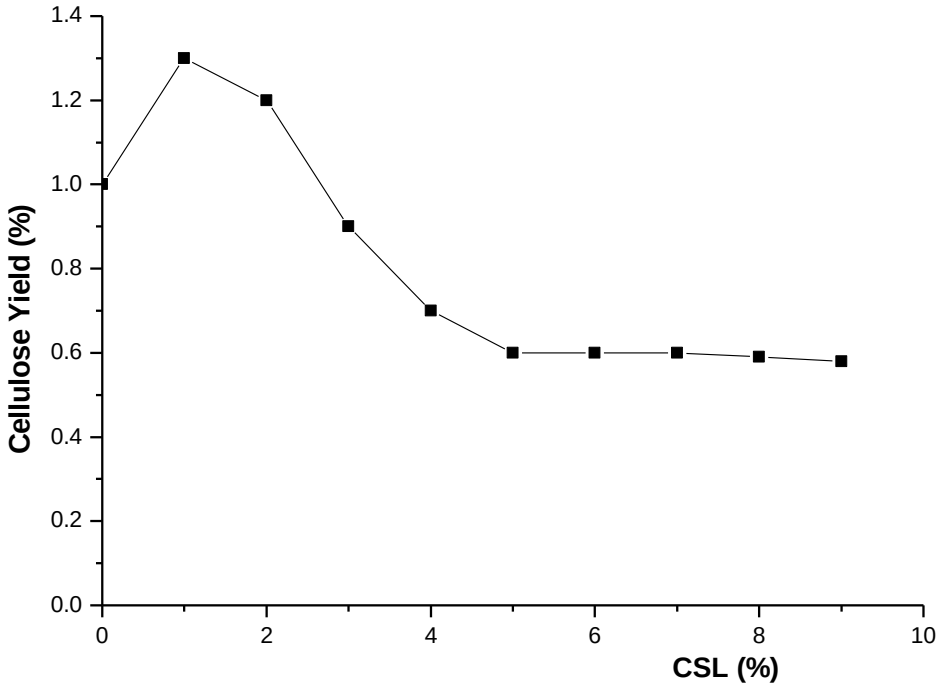


Fig. 4. Effect of CSL on cellulose production using tea extract media (1.0 g/100 ml) containing 20% sucrose for 10 days

Effect of various additives i.e. *p*-aminobenzoic acid, folic acid, lactic acid, nicotinic acid and glycerol on cellulose production was examined. The pH values, at the end of the fermentation period, ranged from 1.8 to 2.2 in all experiments. The control experiment involves cultivating Kombucha organisms in a tea broth (tea extract 1.0 g / 100 ml H_2O) containing sucrose (20%) and CSL (1.0%) for 10 days. The yield was 1.30 g / 100 ml culture. Results presented in **Fig. (5)** showed that folic acid and *p*-aminobenzoic acid were the only additives that gave positive effect on cellulose yield. Both additives, at the same concentration (0.20), gave similar yields being 2.37 and 2.43% respectively **(Table 1)**. *p*-Aminobenzoic acid is a building block of folic acid and incorporates in its biosynthesis. Folic acid is known as a growth factor for some microorganisms (**Varley *et al* 1991**).

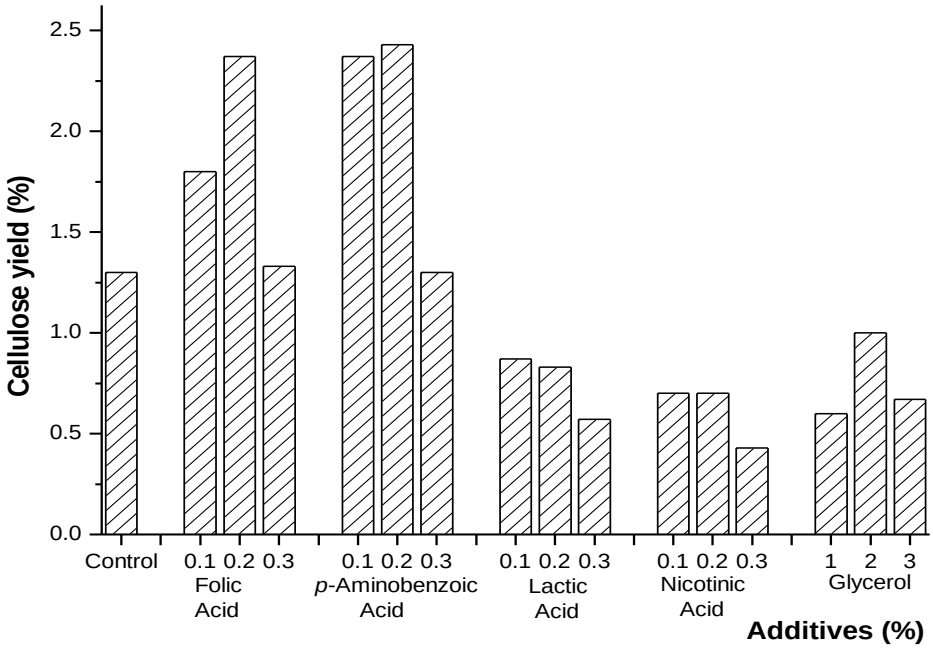


Fig. 5. Effect of some additives on microbial cellulose production

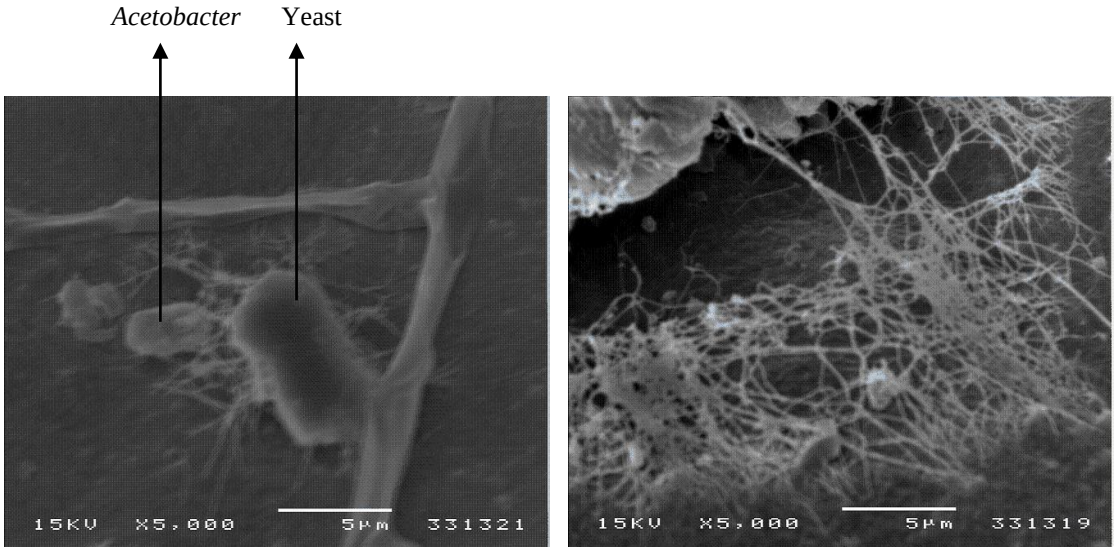
Table 1. Effect of some additives on microbial cellulose production

Additive	Concentration (%)	% Yield (± SD)
No additive (control)	-	1.30 (± 0.10)
Folic acid	0.1	1.80 (± 0.60)
	0.2	2.37 (± 0.21)
	0.3	1.33 (±0.42)
p-Aminobenzoic acid	0.1	2.37 (±0.06)
	0.2	2.43 (±0.15)
	0.3	1.30 (±0.50)
Lactic acid	0.1	0.87 (±0.06)
	0.2	0.83 (±0.15)
	0.3	0.57 (±0.25)
Nicotinic acid	0.1	0.70 (±0.10)
	0.2	0.70 (±0.30)
	0.3	0.43 (±0.32)
Glycerol	1.0	0.60 (±0.10)
	2.0	1.00 (±0.10)
	3.0	0.67 (±0.06)

Scanning electron micrograph **(Fig. 6A)** indicated that the microbial flora of Kombucha (yeast and *Acetobacter*) was embedded in cellulose mat, before NaOH treatments; while **Fig. (6B)** showed the produced microbial cellulose fibrils obtained after treatment without any microbial flora.

CONCLUSION

The present condition for microbial cellulose production involves using available commercial kombucha microorganisms and economic medium (tea broth) that contains low price carbon (sucrose) and nitrogen (CSL) sources. The yield (1.30 g /100ml) was higher than some reported values e.g. 0.661 g /100 ml (**Yang *et al* 1998**). The productive rate (1.3 X 10^{-3} g / day / ml) was also higher than that reported by **Jonas and Farah (1998)**, 1.0 X 10^{-3} g / day /ml, and references therein. Addition of *p*-aminobenzoic acid led to doubling the yield



(A) (B)

Fig. 6. Scanning electron micrograph of untreated (A) and treated (B) microbial cellulose fibril with 1% NaOH

to 2.43 g /100 ml and the productive rate to 2.43 X 10^{-3} g / day / ml. Therefore, using commercial Kombucha, under our condition, for microbial cellulose production could be more economic and give better yield than those reported in some other investigations.

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أنتاج السليولوز الميكروبى بفطر الشاى "الكمبوشا"

[٤]

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لأنتاج سليولوز ميكروبى إقتصادى تم إستخدام بادئ فطر الشاى التجارى "الكمبوشا" المنخفض التكاليف و المكون بصفة أساسية من بكتريا الأسيتوباكتر و فطر الخميرة، و ذلك بتقنية المزرعة الثابتة. و قد وجد أن أفضل بيئة للأنتاج هى مستخلص الشاى الأسود (١ جم/١٠٠ مل) المحتوى على ٢٠% سكروز و ١% منقوع الذرة عند ٢٦- ٢٨°م لمدة ١٠ أيام حيث أعطت ناتج من السليولوز ٣و١ جم/١٠٠ مل بيئة و معدل إنتاج ٣و١X١٠$^{-٣}$ جم/يوم/مل وهو أعلى مما سبق تسجيله فى بعض الأبحاث السابقة. و قد وجد أن أضافة حمض الفوليك أو بارا أمينو حمض البنزويك بتركيز ٢و % ضاعف تقريبا ناتج السليولوز الى ٣٧و٢ و ٤٣و٢ جم/١٠٠مل و معدل الأنتاج الى ٣٧و٢X١٠$^{-٣}$ و ٤٣ و٢X١٠$^{-٣}$ جم/يوم/مل على الترتيب. كما أوضح التصوير بالمجهر الألكترونى الماسح تركيب السليولوز الناتج و أنه خالى من أفلورا ميكروبية بعد المعاملة بواسطة ١% أيدروكسيد صوديوم.

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