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Bacterial Cellulose Production by *Acetobacter xylinum* Strains from Agricultural Waste Products

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Abstract Bacterial cellulose is a biopolysaccharide produced from the bacteria, *Acetobacter xylinum*. Static batch fermentations for bacterial cellulose production were studied in coconut and pineapple juices under 30 °C in 5-l fermenters by using three *Acetobacter* strains: *A. xylinum* TISTR 998, *A. xylinum* TISTR 975, and *A. xylinum* TISTR 893. Experiments were carried out to compare bacterial cellulose yields along with growth kinetic analysis. Results showed that *A. xylinum* TISTR 998 produced a bacterial cellulose yield of 553.33 g/l, while *A. xylinum* TISTR 893 produced 453.33 g/l and *A. xylinum* TISTR 975 produced 243.33 g/l. In pineapple juice, the yields for *A. xylinum* TISTR 893, 975, and 998 were 576.66, 546.66, and 520 g/l, respectively. The strain TISTR 998 showed the highest productivity when using coconut juice. Morphological properties of cellulose pellicles, in terms of texture and color, were also measured, and the textures were not significantly different among treatments.

Keywords Bacterial cellulose · *Acetobacter xylinum* · Texture · Coconut juice · Pineapple

Introduction

Cellulose is composed of the homopolymer of β-1, 4-linked D-glucose. The degree of polymerization of cellulose varies from 100–15,000 glucose units with the crystallization of the long linear chains to form microfibrils of a single crystalline entity [1, 2]. Relatively pure cellulose is produced by the bacteria *Acetobacter xylinum*. This microorganism has been studied for more than 100 years. Unlike the cellulose from wood pulp, bacterial cellulose is devoid of other contaminating polysaccharides such as lignin and hemicellulose, and its isolation and purification are relatively simple, not requiring energy- or chemical-intensive processes [3]. This bacterium has been used as the model system of choice in the exploration of the processes of biogenesis [4–6]. Although the process of formation of cellulose by *A. xylinum* had been investigated quite extensively in earlier studies, most investigations have dealt with the elucidation of cellulose biosynthesis [7–10],

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and most strains studied so far produce only a smaller amount of pellicle under nonagitated conditions. Moreover, a standard medium used for the cultivation of bacterial producing organisms, the Hestrin–Schramm medium [11], is expensive and requires many additional resources for cultivation.

Polysaccharide-producing microorganisms are simple and capable of constructing a polymer from available raw materials and secondary raw material sources. Products from beets (molasses, sugar syrup, and saccharose), corn (starch, hydrolyzed starch, glucose syrup, and glucose), and potatoes (starch and starch hydrolyzates) can be used for producing such polymers. Inedible substrates such as peat hydrolyzates, wood, dextran production wastes, petrochemical wastes and products, ethanol, methanol, glycerin, and ethylene glycol are often suitable [12]. Coconut and pineapple are popular fruits grown in many tropical countries, and they are available over a large part of the year. Coconut juice is mostly discarded as waste from various agro-industries. Since the juices are rich in carbohydrates, proteins, and trace elements, they can be used as a substrate for the production of food grade bacterial cellulose, and also as a raw material for a pure quality paper. Although these two substrates have been used for the traditional cultivation of bacterial cellulose in Southeast Asia, an efficient medium composition has never been reported. Therefore, the main objective of this study was to develop a simple and relatively inexpensive fermentation process for the production of bacterial cellulose employing static batch fermentation with nonconventional agro-residues. Comparison of bacterial strains, growth kinetics, and parameters related to cellulose production were also investigated.

Materials and Methods

Culture Media and Conditions

Three different strains of *A. xylinum*: *A. xylinum* TISTR 998, *A. xylinum* TISTR 975, and *A. xylinum* TISTR 893 were obtained from the Thailand Institute of Scientific and Technological Research, Bangkok, Thailand. Inoculum preparations were transferred from the stock solution to Yeast Malt extract agar slants and incubated for 2 days at 30 °C before being transferred to 5 ml of immature coconut juice to accelerate the growth for a week and then scaled up to 50 ml with the same substrate. The filtrated juices from commercially available coconut and pineapple juices were sterilized before cultivation. A 10% portion of the total volume of each inoculum media was then inoculated into the mature sterilized coconut and pineapple juices with 1% yeast extract supplementation and 14 ml of 95% ethanol in 500 ml for 6 days at 30 °C at a pH of 4.75. Scaling up to 5,000-ml cultivation, at the same pH of sterilized coconut and pineapple juices, was accomplished by static culturing for 2 weeks in a 5-l container to allow for pellicle formation. Cultures were grown in triplicate and repeated twice. Bacterial cellulose pellicles grown on the liquid surface were collected and washed overnight with running water and then immersed in 1 N NaOH for 2 days at 30 °C to dissolve the cells in the pellicle. The pellicles were then immersed under distilled water with 0.02% NaN_2 to reduce microbial contamination and then kept under 4 °C.

Analytical Methods

Samples were collected at regular intervals for 2 weeks of fermentation to quantify cell mass, substrate consumption, and bacterial cellulose production. The cells were collected after centrifugation at 9,200×*g* force for 30 min at 4 °C. The cell mass was estimated by

measuring the optical density at 600 nm after treating the culture broth with 5% cellulase (Celluclast 1.5 l, Novozyme A/S, Bagsvaerd, Denmark) at 50 °C for 30 min. Dry weight determinations for the dry cell mass were also measured. The supernatant was collected to determine residual sugar content by using the Anthrone method [13]. To measure the total cellulose produced, cellulose in the culture broth was washed twice with distilled water and then treated with 1% NaOH at 90 °C for 30 min to dissolve the cell mass. Purified cellulose was washed twice with distilled water and weighed. Total acidity and pH of the medium were also measured over the course of the fermentation. The growth kinetics were reported in terms of growth rate (r_x, g/l/h), specific growth rate (μ, h^{-1}), rate of substrate utilization (r_s, g/l/h), rate of product formation (r_p, g/l/h), specific rate of substrate utilization (Q_s, g/g/h), and specific rate of product formation (Q_p, g/g/h).

Total Viable Count

The number of viable cells in the inocula was determined at timed intervals by the pour plate technique with HS medium. Colonies were counted after 5 days of incubation at 30 °C.

Texture Evaluation

Bacterial celluloses, sized 7.5×7.5×1 cm, were used after incubation in a hot oven at 100 °C for 3 h. Texture was analyzed with a texture analyzer (TA-XT21, Memmert, Germany) with Crip Sracture Rig (HDP/CFS). The instrument was set as follows: 4 cm distance between probe and samples, 1.0 mm/s pretest speed, 1.0 mm/s test speed, and 10.0 mm/s post-test speed. The compression test was run at a rate of 20 times per sample. The compression force was reported.

Color Analysis

A section of 10 g of bacterial cellulose was cut into 1 cm^3 sections to measure color appearance. The values of L^*, a^*, and b^* were measured by a Hunter Lab Color Quest (Memmert, Germany) colorimeter with the CIELAB color system. These values were then used to calculate chroma (C^*) and hue angle (h_{ab}) values. L^* indicates lightness, with a scale ranging from 0 (black) to 100 (white). Positives and negatives in a^* represent red and green, whereas positives and negatives in b^* represent yellow and blue, respectively.

Statistical Analysis

Each treatment was conducted in triplicate and all experiments were repeated at least twice. The statistical significance of the evaluated data was analyzed by one-way analysis of variance. Differences among the mean values were tested using the least significant difference multiple range test. Values were considered significant when $p<0.05$, except when otherwise indicated.

Results and Discussion

Cell Growth and Substrate Utilization in Different Strains

This study aimed to select the best strain for bacterial cellulose (BC) production along with the investigation of the cell growth and substrate utilization. The kinetics of growth and

substrate conversion during BC production by all three strains are shown in Table 1. It was found that TISTR 998 produced BC at approximately a twofold higher rate than the rates of both TISTR 893 and 975. Although the specific growth rate of biomass revealed the same values for all strains, with no significant difference among TISTR 998 with 975 and 893 (p=0.139 and 0.165), TISTR 998 showed a higher efficiency of metabolization for sucrose, glucose, and fructose in coconut juice, and conversion into BC with a value of specific rate of substrate utilization that was 2.75-fold higher than that for TISTR 975. There was a significant difference between the substrate utilization rates of TISTR 998 and 975 (p= 0.0066). This resulted in highest specific rate of product formation (6548.33 g/g/h) that was obtained. Figure 1, which includes values from which the values of Table 1 were calculated, illustrates the bacterial growth curve as the consumption of substrate occurred. All three strains grown in coconut and pineapple juice had a similar pattern of increase in biomass concentrations with decreasing substrate concentrations (Figs. 1 and 2), which is supported by the similarity of growth rates that were seen in Table 1. A rapid increase in cell growth during the 8-day period of fermentation was found for all strains cultured in coconut juice.

Table 1 summarizes the fermentation kinetics of the strains grown on pineapple juice. The final yield of BC from all strains resulted in approximately the same concentration. Both TISTR 893 and 975 showed higher values of specific rate of substrate utilization than TISTR 998, while the growth rates of all strains showed the same profile (Fig. 2). There was, however, a significant difference in substrate utilization when TISTR 998 is compared with both 893 and 975 (p=0.091, p=0.210 respectively). For pineapple, TISTR 893 showed the highest level of BC production, which was different from coconut juice, as TISTR 998 yielded the highest BC production. The changes over the time course of fermentation (Fig. 3) also showed an obvious slower rate of increasing BC after the 6 days of TISTR 975 cultivation, as can be seen in the lower cellulose yield. This result provides strong support for the notion that the static culture method for BC production is more suitable for commercial-scale production, as higher production rates can be achieved. Our strategy for using strains capable of producing cellulose with a high yield have been accomplished, as shown in the high specific rates of product formation (Q_p values, Table 1). This approach is the easiest way to culture BC, with a low production cost, and without extreme attempts to perform chemical mutations and gene modifications to obtain potent strains.

Bacterial Cellulose Production in Different Strains

In a static culture condition, the amount of BC produced gradually increased when produced on coconut juice, these values were 21.85, 18.24, 13.52 cm^3/l per day of TISTR

Table 1 The growth kinetic parameters during the static fermentation of *Acetobacter xylinum* strains in coconut and pineapple juice at 30 °C for 2 weeks.

Substrate	Strains	Bacterial cellulose (g/l)	r_x (g/l/h)	μ (h^{-1})	r_s (g/l/h)	Q_s (g/g/h)	r_p (g/l/h)	Q_p (g/g/h)
Coconut juice	998	553.33	0.041	0.50	3.48	42.96	530.42	6,548.33
	893	453.33	0.045	0.50	1.52	17.12	191.20	2,148.32
	975	243.33	0.050	0.50	1.56	15.57	155.74	1,557.39
Pineapple juice	998	520.00	0.002	0.021	2.871	25.78	213.60	2,080.24
	893	576.66	0.002	0.022	2.922	34.24	401.24	4,702.08
	975	546.66	0.002	0.021	2.734	34.46	434.38	5,475.32

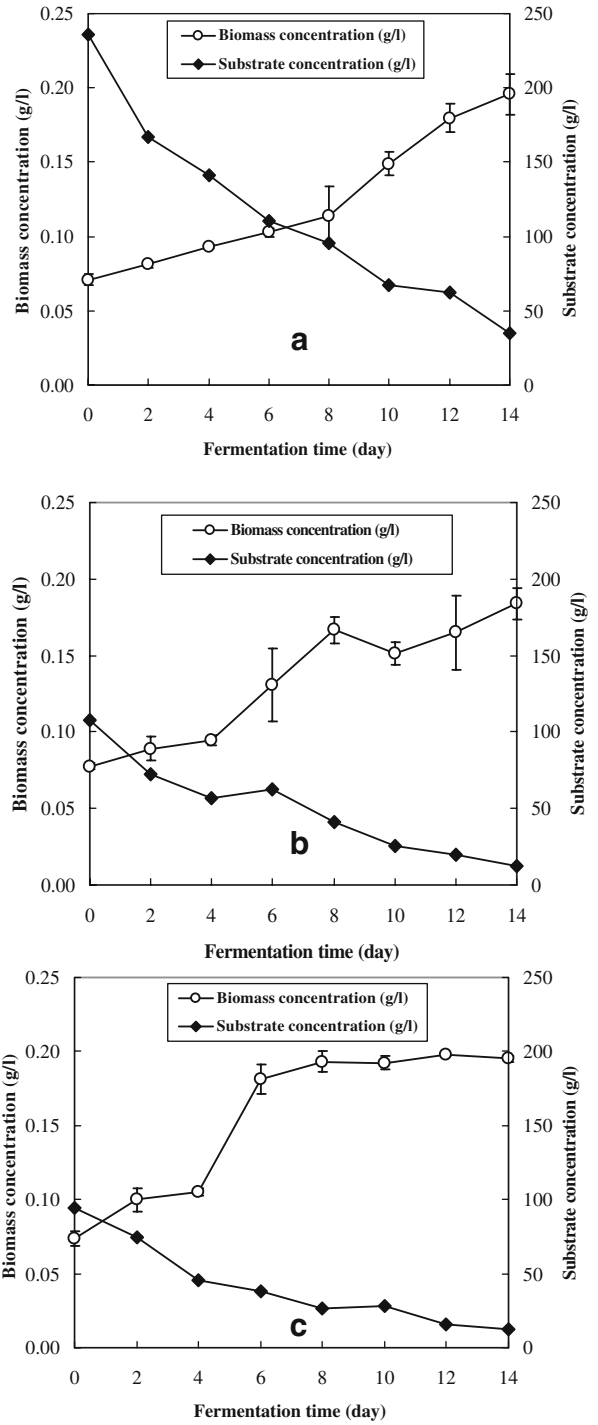

Fig. 1 Time course of cell growth and substrate utilization using *Acetobacter xylinum* grown in coconut juice, **a** TISTR 998, **b** TISTR 893, and **c** TISTR 975. *Values* represent the mean of triplicate determination; *error bars* represent ±standard deviation. When not shown, the error bars fall within the symbols

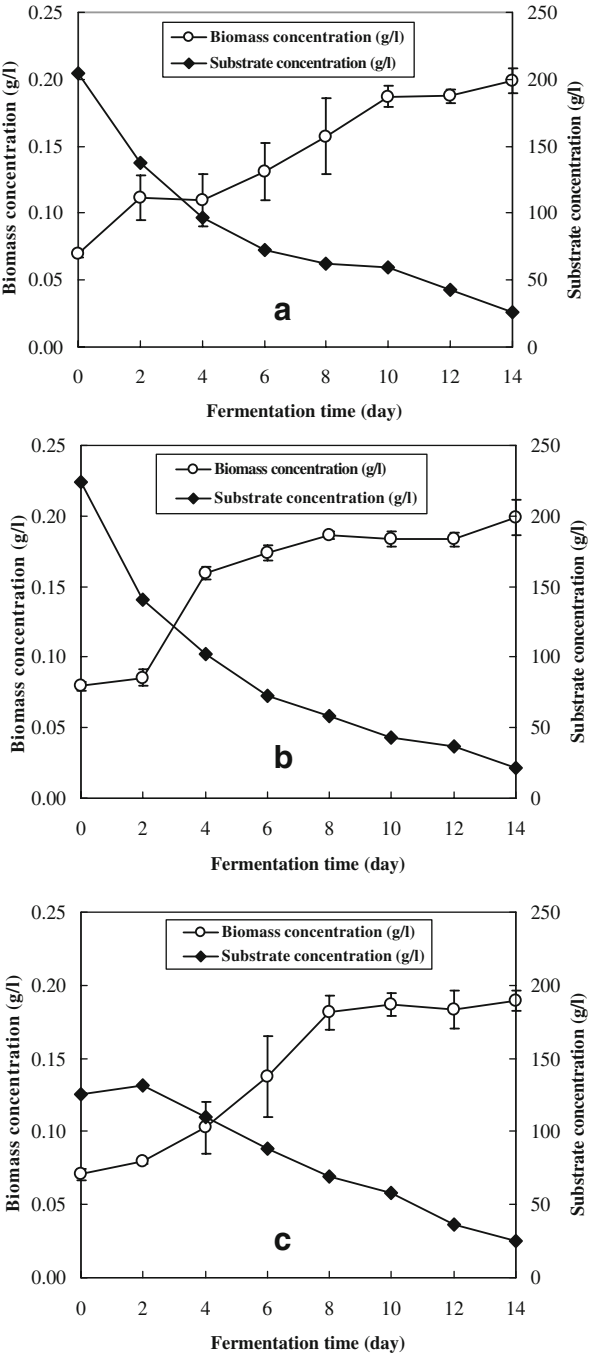

Fig. 2 Time course of cell growth and substrate utilization using *Acetobacter xylinum* grown in pineapple juice, **a** TISTR 998, **b** TISTR 893, and **c** TISTR 975

998, 893, and 975, respectively (Fig. 3a). Cellulose produced in this static condition occurred as a big oval pellicle with a diameter of 26 cm and with a thickness between 2.7 and 3.6 cm. Rates of BC formation on pineapple juice are illustrated in Fig. 3b. Bacterial strains TISTR 998, 893, and 975 produced cellulose at rates of 8.96, 19.02, and 15.25 cm^3/

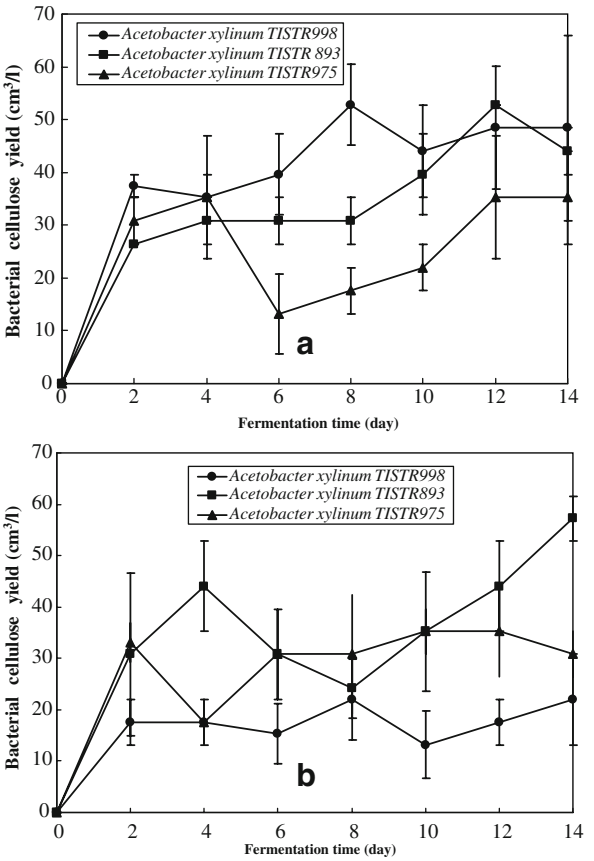

Fig. 3 Rates of bacterial cellulose production during the course of fermentation on **a** coconut juice and **b** pineapple juice

l per day, respectively. In contrast to an agitated culture, *A.xylinium*, a gram-negative, obligate aerobic, rod-shaped organism, produces cellulose at the air–water interface as an assembly of highly crystalline interwoven ribbons that are chemically pure, free of lignin and hemicellulose, and have a high degree of polymerization. The cells and fibrils of cellulose attach to the surfaces of the disks to form tough gelatinous mats that become limited in thickness only by the distance to the adjacent disk. The majority *A. xylinum* cells are found at the top of the growing pellicle, where cellulose production takes place. Since new cellulose is produced at the surface, the pellicle is formed in a downward direction. The steady increase in bacterial cellulose yield in both substrates reveals a spontaneous appearance of cellulose non-producing mutants, and a serious clump-forming problem, which is normally found in an agitated culture. As a consequence of this disturbed condition, a decline in cellulose yield as well as a non-uniform structure may occur.

Viable Cell Growth in Different Strains

The changes in total viable cell count during the course of the fermentations are given for both juices in Fig. 4a,b. Bacterial growth exponentially increased, reaching 10^{11} times the value of the initial day after 8 days of cultivation (Fig. 4a,b). Both substrates showed similar curves of cell growth and the curves approached stationary growth after 8 days of fermentation time. Both substrates clearly showed two phases of cell generation that had

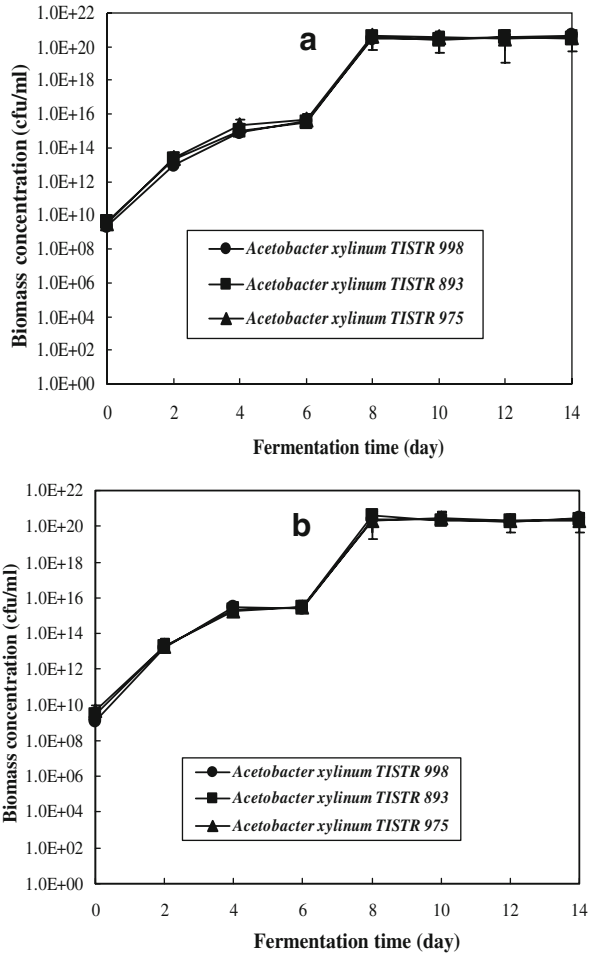

Fig. 4 Viable cell count during the fermentation of bacterial cellulose on **a** coconut juice and **b** pineapple juice

both exponential and stationary growth phases during the fermentation time. There was no cell death phase during this period because of the substantial amount of substrate still remaining in the fermentation broth. Cellulose levels increased the whole time due to continuous cell growth. The doubling time of this type of bacteria is known to be within the range 1.5 to 8 h [14], and our data showed a doubling time of 2 h. The growth rate of fibrils is not well-known, but a rate of ~2 μm min^{-1} has been reported as the rate observed for isolated cells during the initial stage of cultivation [15].

Total Acid and pH Changes During Fermentation

As fermentation time increased, there was an increase in the total acid observed (as measured by the presence of acetic acid secreted during bacterial growth and cellulose production, Fig. 5a,b). This is because *A. xylinum* is unique in its family for being able to convert carbohydrates to acetic acid by synthesizing and extruding fibrils of cellulose. Its metabolism is respiratory, which involves oxidizing ethanol to acetic acid and converting glucose to gluconic acid. Acetic acid is a by-product of cellulose, which influences the

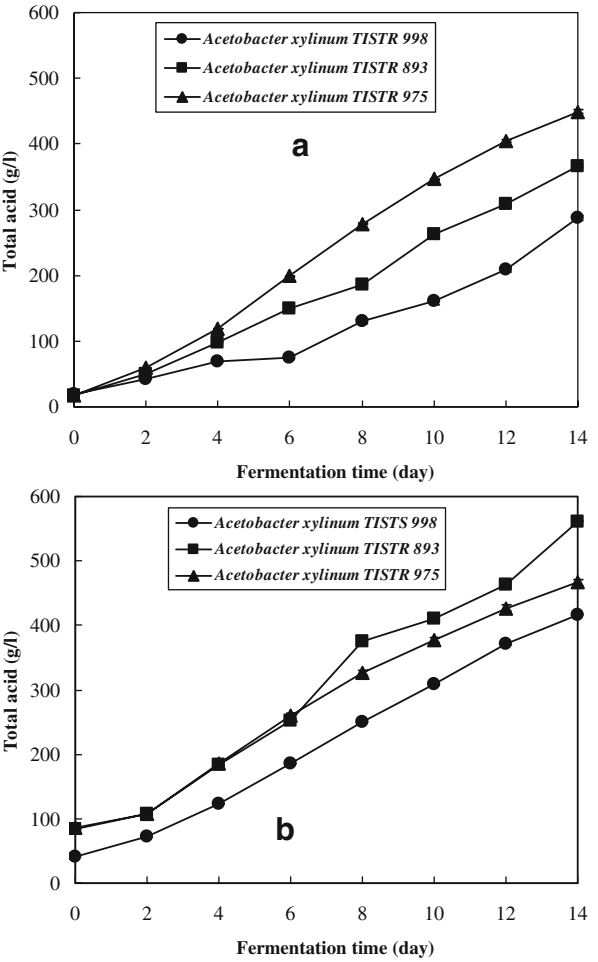

Fig. 5 Total acid production from the fermentation by three strains of *Acetobacter xylinum* of **a** coconut juice and **b** pineapple juice

decreased pH in the culture medium. As proposed by Seto et al. [16], a decrease in pH of the culture medium has an effect on both cellulose production and cell growth for *A. xylinum*. The pH of both substrates had changed to lower the acidity (Fig. 6a,b). While bacterial cellulose is being produced, this change in acidity can be difficult to control by the pH buffer system, as has been observed by some investigators [17, 18]. This pH reduction was also observed in the Hestrin–Schramm medium when cultured with the same strains (TISTR 893,975) in molasses, as reported by Premjet et al. [19]. When coconut juice was the substrate, the highest acetic acid concentration was found in TISTR 975, with a 24.96-fold increase (Fig. 5a). Acetic acid content at levels of about 20.37-fold and 14.58-fold were detected in TISTR 893 and 998, respectively. For pineapple juice (Fig. 5b), TISTR 998, 893, and 975 produced acetic acid at levels of increase of 10-, 6.68-, and 5.47-fold, respectively. The only significant difference in total acid production between substrates for a given strain was seen in the higher rate of production by TISTR 975 over that of 998 on pineapple substrate (Fig. 5b). This value was also significantly greater than the values seen for the other strains (Fig. 5a).

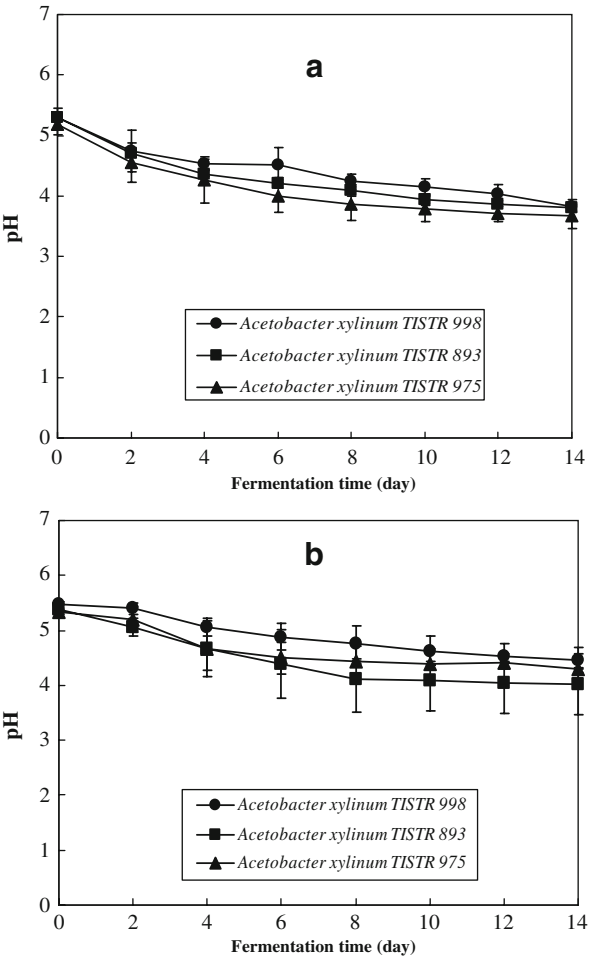

Fig. 6 Time course of pH change during fermentation using **a** coconut juice and **b** pineapple juice

Effect of Texture on Bacterial Cellulose in Different Strains

The strengths of the resulting dried sheets were tested by applying mechanical compression forces to determine the relative effects of the bacterial strains. As shown (Table 2), there were no significant differences between the textures of all bacterial cellulose strength levels derived from the three strains. Both coconut and pineapple juices yielded the same strength rating. The mechanical properties of bacterial cellulose, both air-dried and hot-pressed

Table 2 Comparison of the compression forces on bacterial celluloses.

Bacterial strains	Force (kg)	
	Coconut juice	Pineapple juice
TISTR 893	0.086±0.043	0.076±0.016
TISTR 975	0.072±0.013	0.090±0.038
TISTR 998	0.070±0.022	0.073±0.037

Table 3 Color comparison of bacterial cellulose.

Substrate	Bacterial strain	CIELab values (standard error)				
		L^*	a^*	b^*	C^*	h_{ab}
Coconut juice	893	52.46 (1.67)	−2.45 (0.10)	−0.47 (0.60)	2.78 (0.08)	194.02 (13.70)
	975	53.62 (2.78)	−3.33 (0.03)	0.13 (0.64)	3.58 (0.29)	195.69 (19.85)
	998	53.02 (1.37)	−3.05 (0.18)	1.18 (0.72)	3.59 (0.17)	158.41 (13.32)
Pineapple juice	893	47.17 (0.99)	0.01 (0.31)	13.30 (0.49)	13.00 (0.63)	88.40 (0.75)
	975	54.00 (1.41)	2.12 (0.33)	19.41 (0.11)	19.22 (0.79)	83.62 (0.57)
	998	52.46 (1.54)	−2.45 (0.20)	−0.47 (0.70)	2.78 (0.36)	194.02 (0.25)

preparations [20], also showed that there were no differences detected on BC among different bacterial strains. Comparison of two bacterial strains (ATCC 10821 from the American Type Culture Collection, Maryland, USA and AJ 12368 from Central Research Laboratories, Ajinomoto, Tokyo, Japan) did not show any substantial difference (data not shown). The lack of significant differences in mechanical effects among strains was also observed among varying cultivation times and cellulose contents. The experiment depicted in Table 3 also emphasized that the sheets obtained from the pellicles of different strains have the same mechanical properties. All of these strains are useful for producing BC for paper making.

Effect of Color on Bacterial Cellulose in Different Strains

As shown in Table 3, all strains yielded an opaque, white, thick pellicle, as the L^* value shifted slightly toward the lightness side. However, the values obtained from strains 893 and 975 detected higher values of positive b^*, indicating yellowness. Values from the calculated chroma and hue angle for strains 893 and 975 are also shown in Table 3. The samples cultivated on pineapple juice yielded an opaque yellow color, which contained a carotene color, (h_{ab} values approach to 90°, Table 3), while those bacterial celluloses derived from the coconut juice showed a white color.

Conclusions

This study showed that the bacterial cellulose derived from coconut and pineapple juices can be converted efficiently to bacterial cellulose by the supplementation of yeast extract and ethanol under static fermentation conditions at 30 °C. Bacterial celluloses produced from all strains are growth associated products. Coconut juice seems to be a better substrate than pineapple juice. In view of energy consumption, the productivity of BC on this medium is high, which makes the production costs lower than expected. It is also clear that different *A. xylinum* strains produce different BC content levels under the same inoculation volumes and under static cultivation conditions. These results suggest that bacterial cellulose pellicles of all strains appear to be easily applied to use in many applications such as food, paper, and textile industries, without requiring additional steps of decolorization and purification. Furthermore, the properties of cellulose, in terms of crystallinity, high water-absorption capacity, and mechanical strength of the reported strains, have additional applications in cosmetics and medicine.

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