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Thin stillage supplementation greatly enhances bacterial cellulose production by *Gluconacetobacter xylinus*

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ABSTRACT

Thin stillage (TS), a wastewater from rice wine distillery can well sustain the growth of *Gluconacetobacter xylinus* for production of bacterial cellulose (BC). When used as a supplement to the traditional BC production medium (Hestrin and Schramm medium), the enhancement of BC production increased with the amount of TS supplemented in a static culture of *G. xylinus*. When TS was employed to replace distilled water for preparing HS medium (100%TS–HS medium), the BC production in this 100%TS–HS medium was enhanced 2.5-fold to a concentration of 10.38 g/l with sugar to BC conversion yield of 57% after 7 days cultivation. The cost-free TS as a supplement in BC production medium not only can greatly enhance the BC production, but also can effectively dispose the nuisance wastewater of rice wine distillery.

1. Introduction

Thin stillage (TS) or distillery wastewater is the liquid portion of distillery stillage from the fermentation of grain-based feedstock. Its volume is approximately 10 times that of ethanol produced. Thin stillage from rice wine distilleries is rich in carbon sources and organic acids (Hsieh, Hsu, & Yang, 2005; Yang & Lin, 1998). But its low pH and high biological oxygen demand make TS a nuisance to distillery industries. Although TS can be effectively treated by active sludge or anaerobic digestion, from the resource recovery point of view, TS is better to be considered as nutrients rather than wastewater. Thus, many studies have been carried out to ferment TS for the production of value-added product such as ethanol, enzymes, biomass and among others (Ahn, Sang, & Um, 2011; Egg, Sweeten, & Coble, 1985; Yang & Lin, 1998; Yang & Tung, 1996).

Bacterial cellulose (BC) is usually produced by *Gluconacetobacter xylinus* (formerly *Acetobacter xylinum*) as a white leather-like pellicle at the air–liquid interface. The BC pellicle is characterized by a 3D structure consisting of an ultra-fine network of cellulose nanofibers (3–8 nm). Its unique nano-morphology results in a very high water-holding capacity, great elasticity, high wet strength, and conformability. These unique properties as well as its purity enable many successful applications in the field of biomedical materials in recent time (Czaja, Young, Kawecki, & Brown, 2007; Klemm et al., 2011; Petersen & Gatenholm, 2011). Various sugars can be utilized by *G. xylinus* to produce BC, but with a low conversion yield (Bae, Sugano, & Shoda, 2004; Jung et al., 2010). In order to compensate its low sugar conversion yield and to reduce the feed-stock cost of BC production, BC has been produced by fermenting the hydrolysate of agricultural waste such as hemicelluloses (Hong, Zhu, Yang, & Yang, 2011), konjac glucomannan (Hong & Qiu, 2008), rice bark (Goelzer, Faria-Tischer, Vitorino, Sierakowski, & Tischer, 2009) and waste cotton fabrics (Kuo, Lin, & Lee, 2010). In addition to various sugars, ethanol (Park, Jung, & Park, 2003), glycerol (Jung et al., 2010; Mikkelsen, Flanagan, Dykes, & Gidley, 2009), organic acids (Embuscado, Marks, & BeMiller, 1994; Jung et al., 2010; Wang, Wei, Wei, Wang, & Xiang, 2008), and TS from beer culture broth (Ha et al., 2008) have also been used to culture *Gluconacetobacter* for BC production.

Since the supplementation of organic acids in *G. xylinus* culture medium has been reported to be very effective for the enhancement of BC production (Dudman, 1959; Embuscado et al., 1994; Jung et al., 2010; Wang et al., 2008), in this study TS from rice wine distillery which is rich in organic acids was employed to supplement the traditional BC production Hestrin and Schramm (HS) medium. The aims of this study are twofolds: (1) to investigate whether the supplementation of TS can enhance BC production, (2) to dispose the wastewater of rice wine distilleries as a valuable bioresource. TS supplemented to HS medium with different percentages were employed to culture *G. xylinus* statically. The result shows that 100% TS supplement enhanced BC production to a concentration of 10.38 g/l after 7 days cultivation. In addition, the sugar

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Table 1
The composition of thin stillage from rice wine distillery.

pH	3.5
COD (ppm)	50,920
BOD (ppm)	25,000
Sugar (mg/l)	
Reducing sugar	1120
Glucose	35
Acid (mg/l)	
Total organic acid	3833
Succinic acid	2254
Gluconic acid	1018
Acetic acid	243
Citric acid	161
Malic acid	157
Total amino acid	760
Total nitrogen (mg/l)	1258
Total phosphates (mg/l)	129

to BC conversion yield also increased to 57%. To our knowledge, these high BC concentration and conversion yield have never been reported before in a batch static culture of *G. xylinus*.

2. Materials and methods

2.1. Bacterial strain

G. xylinus (BCRC 12334) obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan) was used as a bacterial cellulose producing strain.

2.2. Medium and cultivation

The basic medium used for growing *G. xylinus* is Hestrin and Schramm (HS) medium containing 20 g/l glucose, 20 g/l peptone, 10 g/l yeast extract, 1.15 g/l citric acid, 6.8 g/l Na_2HPO_4 with pH adjusted to 6.0. Thin stillage (TS) obtained from Taichung Distillery of Taiwan Tobacco & Liquor Corporation (Taichung, Taiwan) was filtrated using a 1.2 μm membrane to remove particulates in the solution. The filtrate was stored at 4 °C for further usage. The composition of this TS (Hsieh et al., 2005; Yang & Lin, 1998; this study) is listed in Table 1. HS medium supplemented with different amount of TS (TS–HS medium) was prepared by dissolving all the ingredients except glucose of HS medium in the TS solution diluted with different amount of deionized water. The pH of resulting medium was adjusted to 6.0 before autoclaving. The amount of glucose in HS medium was separately autoclaved and added to the sterilized TS–HS medium. Inoculums culture was prepared by transferring *G. xylinus* cell suspension stored at −80 °C into HS medium and statically cultivated at 30 °C for 3 days. The statically grown culture was then shaken vigorously to release the attached cells from the cellulose pellicle. The resulting cell suspension of 5 ml was inoculated into a 250 ml Erlenmeyer flask containing 45 ml of culture medium and then statically incubated at 30 °C for 7 days in triplicate experiments.

2.3. Analytical methods

The reducing sugar concentration of culture medium was determined using the dinitrosalicylic acid (DNS) method. The total acid concentration was determined by titration with 0.1N NaOH with phenolphthalein as an indicator. The composition of organic acids in TS was determined by HPLC analysis (Acme 9000 HPLC system, Younglin Instrument Co., Korea) equipped with a Rezex ROA organic acid column (Phenomenex Inc., USA). The cellulose pellicle produced in the static cultures was purified by heating the pellicle in 1N NaOH at 95 °C for 3 h to eliminate the entrapped cells, followed by thoroughly washing with deionized water to neutral pH. The purified cellulose pellicle was dried at 90 °C to a constant weight. The dry cell weight in the culture was determined by collecting the BC pellicle with culture broth by centrifugation at 12,000 × *g* for 10 min. The pellet obtained was suspended in pH 4.8, 0.1 M citrate buffer and 1% (v/v) cellulase (ACCELLERASE 1500, Danisco Inc., USA) was added to degrade the cellulose pellicle. After 200 rpm shaking for 9 h at 50 °C to reach a complete hydrolysis, the digested suspension was centrifuged and washed twice with triple amount of deionized water then dried at 90 °C (Hu & Catchmark, 2010).

3. Results and discussion

3.1. Effect of thin stillage supplementation on BC production

Thin stillage from rice wine distillery is an acidic solution with pH around 3.5. As shown in Table 1, TS is rich in organic acids and amino acids that prompts us to consider it as a potential supplement for growing acetic acid bacteria *G. xylinus* for the production of BC. Since the solid content of HS medium is included in TS–HS medium, the HS ingredients concentrations are same in all TS–HS medium with different percentages of TS supplementation. As shown in Fig. 1a, the final pH of *G. xylinus* culture after 7 days static cultivation was not much affected by the amount of TS supplemented. On the contrary, the total cell weight in the culture increased with the amount of TS employed in TS–HS medium (Fig. 1b). The cell weight increased about 26% from 1.43 g/l to 1.82 g/l as TS supplementation increased from nil to 100%. In addition to the slight cell weight increase, a significant enhancement of BC production from 4.15 g/l to 7.35 g/l was noticed when 12.5% TS was employed. The BC production can be further increased to 10.22 g/l as TS supplementation increased to 100%. Evidently, the supplementation of TS is very effective for enhancing BC production. Since there is a less amount of reducing sugar in TS (ca. 0.1%, w/v), the amount of reducing sugar can be brought in by the increase of TS supplementation will not be possible to generate an approximately 2.5-fold enhancement on BC production. In fact, as shown in Fig. 1a, the reducing sugar consumption decreased slightly with the increase of TS. Based on the total reducing sugar in the medium (ca. 1 g in 50 ml culture), the percentage of reducing sugar consumption declined from 97% to 87% as TS supplementation increased from nil to 100%. The lower reducing sugar consumption but with an enhanced BC production leads to a significant increase of sugar to BC conversion yield from 20% to 57% (Fig. 1b). In other words, 57 g of BC was produced as 100 g of reducing sugar was consumed in 100%TS–HS medium. This indicates that the amount of reducing sugar brought in from TS supplementation did not contribute to the BC production enhancement. Probably, the rich organic acids content in TS (Table 1) can well support the growth of *G. xylinus* so that most of glucose in HS medium can be utilized for BC biosynthesis.

3.2. Time course of G. xylinus growth in TS–HS medium

The time courses of growing *G. xylinus* in HS-only medium, 100%TS–HS medium, and TS-only medium were studied to further confirm the beneficial effect of TS supplement on enhancing BC production. As shown in Fig. 2a, in the HS-only medium, the reducing sugar was rapidly consumed from 1.09 g to 0.12 g after 3 days cultivation of *G. xylinus*. In contrast, as shown in Fig. 2b, the reducing sugar consumption rate was much slower in 100%TS–HS medium that approximately 0.45 g of reducing sugar still can be detected after 3 days cultivation. During the first 3 days cultivation, the consumption of reducing sugar resulted in an apparent pH decrease in both HS-only and 100%TS–HS medium. Besides, the increase of total

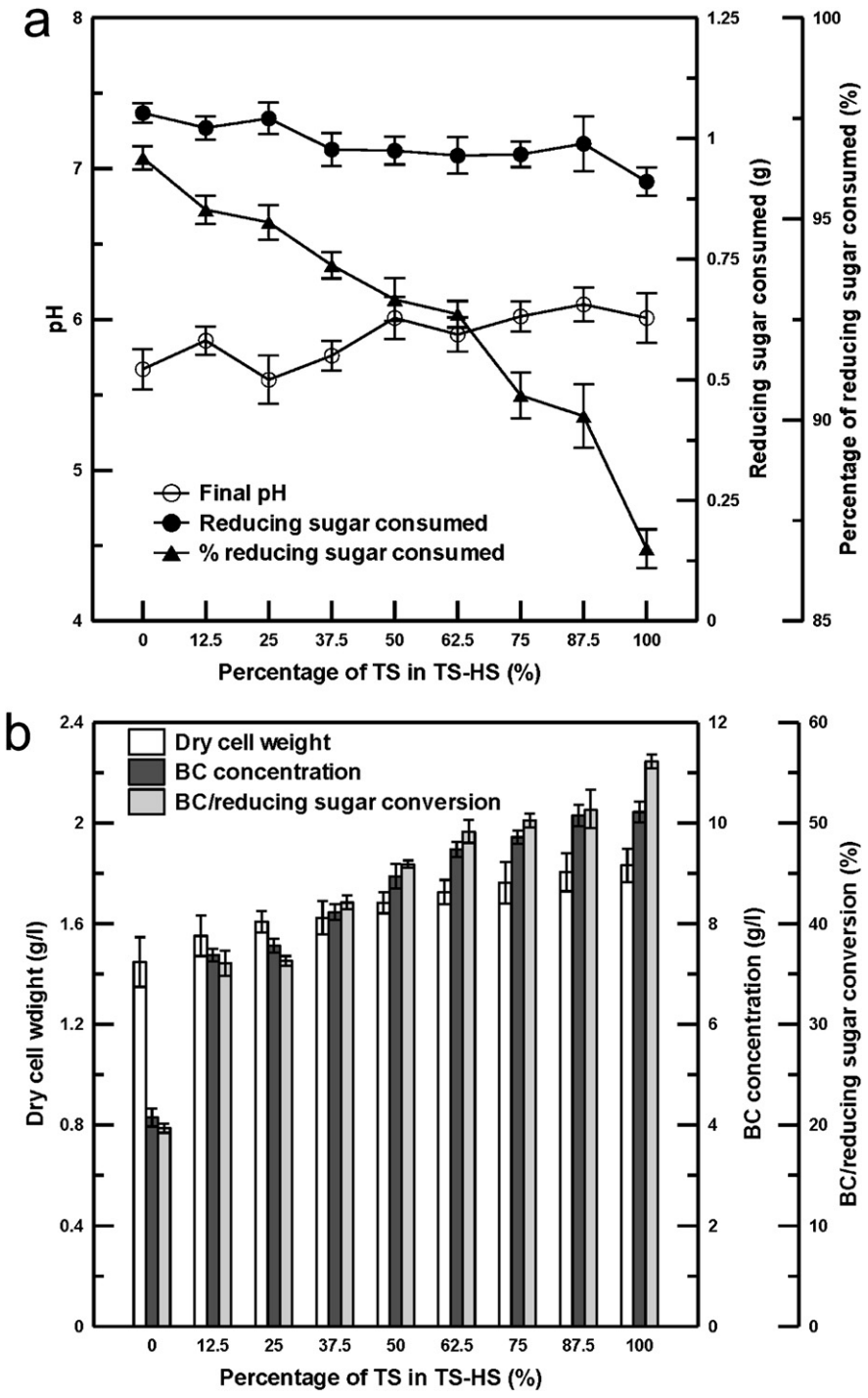

Fig. 1. Effect of different percentage of TS supplemented in HS medium after 7 days cultivation on (a) final pH and reducing sugar consumed; (b) dry cell weight, BC concentration and BC conversion yield.

acids was also observed that well corresponds to the decrease of pH and reducing sugar concentration. However, in the TS-only medium (Fig. 2c), the increase of total acids was also observed but with a slight increase of pH during the first 3 days. Since the reducing sugar was hardly consumed in TS-only medium, the slight increase of pH probably resulted from the release of ammonium ion from metabolizing amino acids in TS by *G. xylinus*. The primary metabolites of the actively growing *G. xylinus* cells may contribute to the increase of organic acids. In these three medium employed, the cell weight of *G. xylinus* increased with cultivation time but with a faster rate during the first 3 days. As cultivation extended over 3 days, the pH of both the HS containing medium started to increase and accompanied by the decrease of total acids. It has been known that the membrane-bound glucose dehydrogenase of *G. xylinus* is very active for converting the extra-cellular glucose into gluconic acid (Shigematsu et al., 2005), therefore, the rapid reducing sugar concentration decrease along with pH decrease and total acids increase observed in both HS containing medium during the first 3 days cultivation evidenced the existence of active glucose dehydrogenase in *G. xylinus*. The produced organic acids will be further utilized for cell growth and BC production when the available reducing sugar declined to a lower level. The *G. xylinus* cell

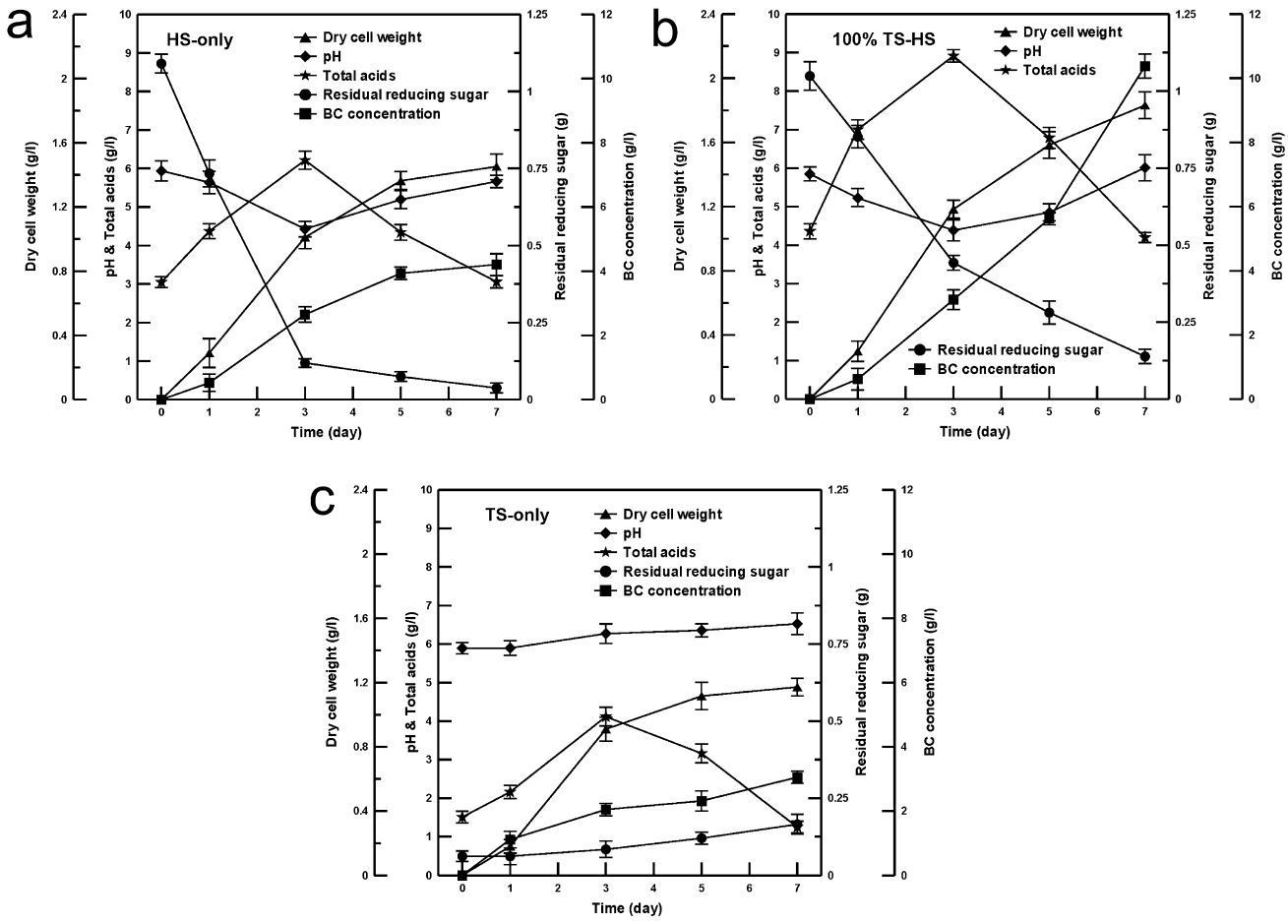

Fig. 2. Time courses of *G. xylinus* growth and BC production statically cultivated at 30 °C, initial pH 6.0 in (a) HS-only; (b) 100%TS–HS; (c) TS-only medium.

density and BC concentration still increased when total acids started to decrease during the last 4 days cultivation. It is worth to note that after 7 days cultivation the TS-only medium can produce 3.05 g/l of BC. The HS-only medium which contains 20 g/l glucose, on the other hand, can only produce 4.21 g/l of BC. Unlike HS-only medium, the BC production rate did not show any decrease in 100%TS–HS medium throughout the 7 days cultivation. The synergetic effect of TS on enhancing BC production could be clearly observed from the 10.38 g/l BC produced in 100%TS–HS medium which is about 2.5- and 3.4-fold higher than that produced by HS-only medium and TS-only medium, respectively. Presumably, the rich organic acids and amino acids in 100%TS–HS medium not only can provide the enough nutrients to support the growth of *G. xylinus* but also has some extent of inhibition effect on transforming glucose into organic acids so that the amount of glucose included in HS medium can be efficiently utilized as building blocks for BC biosynthesis. In order to examine this possibility, the TS–2%D medium that only 20 g/l glucose is added to TS was prepared to culture *G. xylinus* for producing BC. After 7 days cultivation, *G. xylinus* can grow into a density of 1.23 g/l and produce 6.06 g/l of BC in TS–2%D medium (Table 2), however, the TS-only medium can obtain 1.17 g/l of biomass and 3.05 g/l of BC. This indicates that 20 g/l glucose added to TS did not improve cell growth but significantly enhanced 2-fold of BC concentration. That is to say, TS can well support *G. xylinus* growth so that most of glucose supplemented can be utilized for BC biosynthesis. Based on the same amount of glucose, cell weight and BC concentration by *G. xylinus* in TS–2%D medium and HS-only medium were compared. As shown in Table 2, although cell weight in TS–2%D medium after 7 days cultivation was lower than that in HS-only medium, BC concentration in TS–2%D medium was about 1.5-fold higher than that obtained in HS-only medium. This reveals that TS has some extent of inhibition effect on converting glucose into gluconic acid probably due to the second rich gluconic acid (ca. 1 g/l) existed in TS (Table 1) so that glucose can be efficiently used as building blocks for BC biosynthesis. Besides, it is worth to note that the richest organic acid in TS is succinic acid (ca. 2.2 g/l), which has been reported to be a very effective organic acid supplemented for enhancing BC production (Jung et al., 2010). In addition to succinic acid, the other organic acids in TS such as gluconic acid, acetic acid, citric acid and malic acid are also beneficial for BC production enhancement (Jung et al., 2010).

Based on the data obtained from Fig. 2a and b, the reducing sugar consumed and BC conversion yield (based on the reducing sugar) by *G. xylinus* in HS-only medium and 100%TS–HS medium

Table 2
Summary of BC productivity in various medium after 7 days static cultivation.

	HS-only	100%TS–HS	TS-only	TS–2%D
Dry cell weight (g/l)	1.45 ± 0.08	1.83 ± 0.09	1.17 ± 0.06	1.23 ± 0.07
BC concentration (g/l)	4.21 ± 0.29	10.38 ± 0.37	3.05 ± 0.19	6.06 ± 0.35
BC specific productivity (g-BC/g-DCW)	2.90 ± 0.05	5.67 ± 0.07	2.61 ± 0.03	4.93 ± 0.04

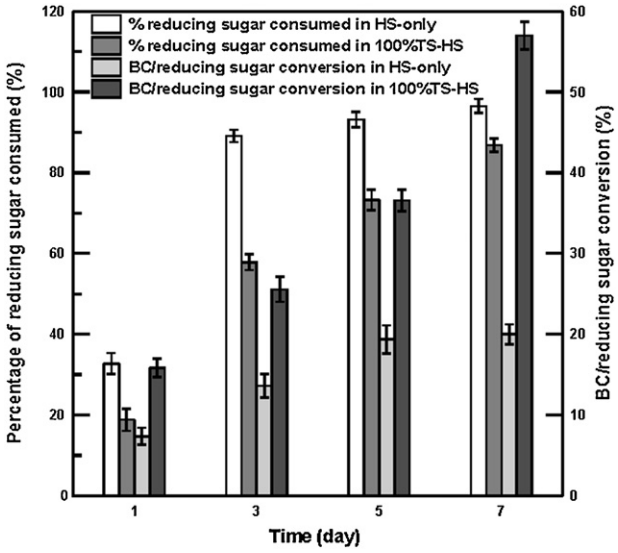

Fig. 3. Comparison of reducing sugar consumed and BC conversion yield by *G. xylinus* after 7 days cultivation in HS-only and 100%TS–HS medium.

were calculated and compared. As shown in Fig. 3, the sugar consumption rate in HS-only medium was much faster than that in 100%TS–HS medium that about 88% reducing sugar was consumed within 3 days while only about 57% reducing sugar was consumed in 100%TS–HS medium. Approximately 85% reducing sugar in 100%TS–HS medium was consumed after 7 days cultivation. With a less sugar consumed but a higher BC production, the BC conversion yield in 100%TS–HS medium was always higher than that in HS-only medium. After 7 days cultivation, the BC conversion yield could reach 57% is about 2.8-fold higher than that obtained in HS-only medium. Table 2 summarizes the specific productivity of *G. xylinus* for BC after 7 days cultivation in the different medium employed. *G. xylinus* could grow into a highest density of 1.83 g/l and produced a highest BC concentration of 10.38 g/l in 100%TS–HS medium. The BC concentration of *G. xylinus* in TS-only medium is about 30% less than that in HS-only medium probably

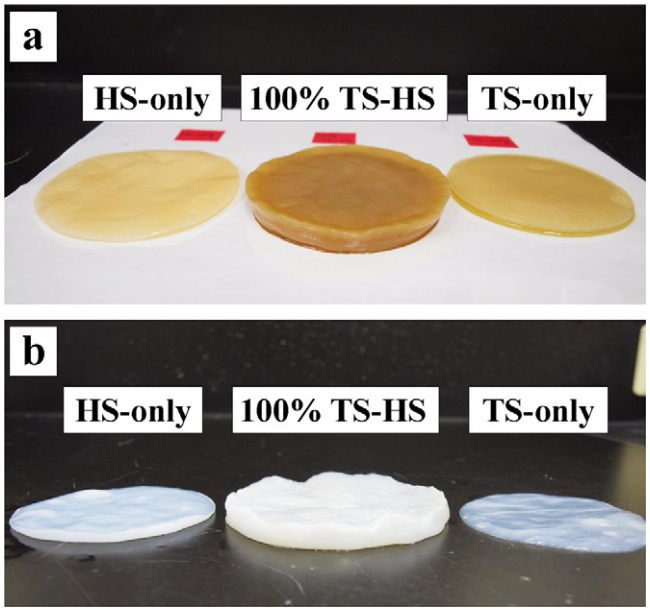

Fig. 4. Optical images of wet BC pellicles obtained from 7 days static cultures of *G. xylinus* in different medium: (a) before; (b) after 1N hot NaOH treatment and thorough deionized water washing.

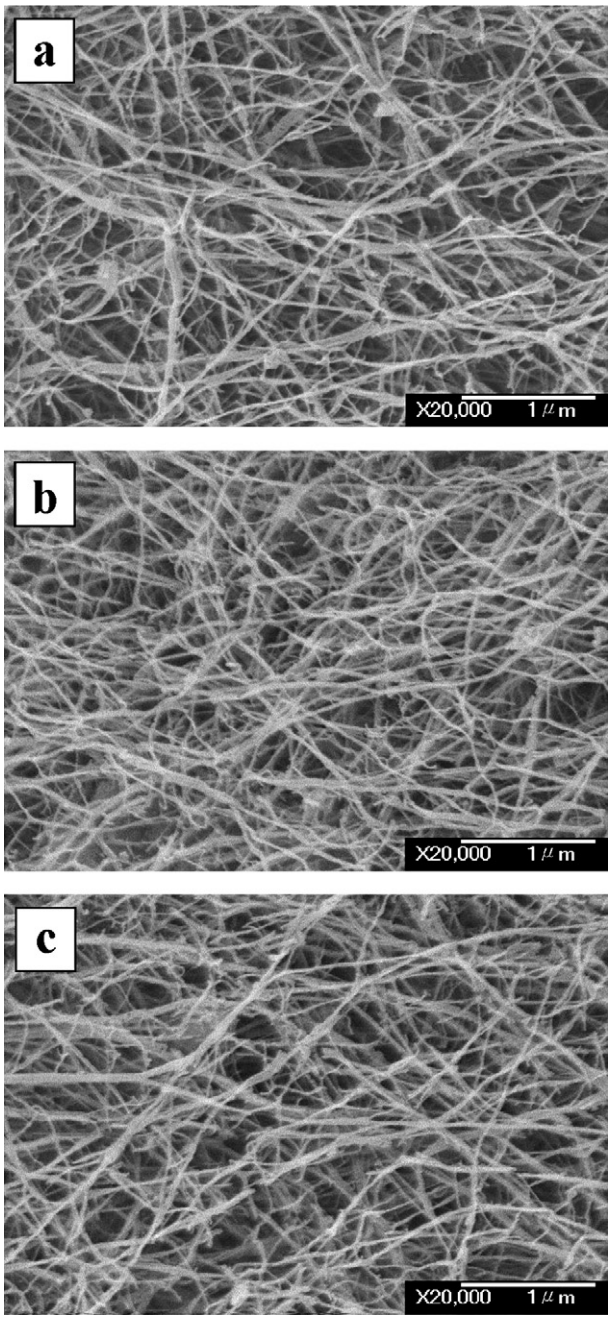

Fig. 5. SEM images of thoroughly washed BC pellicles from 7 days static cultures of *G. xylinus* in (a) TS-only; (b) 100%TS–HS; (c) HS-only medium.

due to the lack of glucose, the readily available building block for BC biosynthesis. The specific productivity of BC in TS–2%D medium was enhanced almost 2-fold to 4.93 g-BC/g-DCW in comparison to TS-only medium. The BC specific productivity in 100%TS–HS medium reached 5.67 g-BC/g-DCW, which is about 2-fold higher than that obtained in HS-only medium. The high BC productivity of *G. xylinus* achieved again shows that TS supplementation can provide sufficient nutrients for *G. xylinus* to grow and the glucose included in HS medium mainly supplied as building blocks for BC biosynthesis.

3.3. Effect of TS supplementation on morphology of BC pellicle

The images of wet BC pellicles obtained from 7 days static cultures of *G. xylinus* in various medium are shown in Fig. 4a. As can

be seen, the BC pellicle produced in 100%TS–HS medium was much thicker than that in HS-only and TS-only medium but with a much deeper color of brownish. The brownish colored pellicle was consisted of culture broth, BC, and the embedded biomass of *G. xylinus*. The deeper color of BC pellicle obtained in 100%TS–HS medium was mainly resulted from the supplementation of TS which is a brownish solution. As shown in Fig. 4b, the original brownish color can be completely removed after treating the BC pellicles with 1N hot NaOH solution and thoroughly washing with deionized water. Based on the thickness of wet pellicles and the weight of dried pellicles, 100%TS–HS medium was demonstrated to be a much better medium for BC production in comparison to HS-only and TS-only medium. Scanning electron microscope (SEM) was employed to observe the micro-structural difference between these BC pellicles. As shown in Fig. 5, all the BC pellicles were consisted of interwoven nanofibers with no significant difference in the fiber size and porosity of the pellicle. Besides, the obtained BC pellicles were subjected to cellulase hydrolysis for the confirmation of their cellulose nature. All three BC pellicles can be completed hydrolyzed by cellulase into clear solutions and only glucose was detected in the hydrolysate by HPLC analysis (see supplemented information). In other words, it can be confirmed that TS can be utilized by *G. xylinus* to produce BC pellicle with same chemical structure and morphology as that produced in traditional HS medium.

4. Conclusions

Thin stillage from rice winery industry was used as a very effective supplement to traditional HS medium for the enhancement of BC production. TS itself supported the growth of *G. xylinus* to produce BC with a concentration of 3.05 g/l after 7 days static cultivation. With inclusion of all the ingredients of HS medium in TS (100%TS–HS medium), the BC production was enhanced 3.4-fold to 10.38 g/l which is about 2.5-fold higher than that obtained in HS-only medium. The presence of TS in HS medium reduced the reducing sugar consumption rate of *G. xylinus* that leaded to a very high BC conversion yield (0.57 g-BC/g-reducing sugar) ever reported. The employment of cost-free TS as a supplement for BC production not only can enhance BC production but also solve the wastewater disposal problem of winery industry.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2012.05.003.

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