Volume 6, Issue 5, 2016, 1450 -1456 ISSN 2069-5837

Biointerface Research in Applied Chemistry

www.BiointerfaceResearch.com

Original Research Article Open Access Journal

Received: 10.05.2016 / Revised: 25.08.2016 / Accepted: 18.09.2016 / Published on-line: 13.10.2016

Evaluation of possibility to produce green biocellulose nanofibers in simultaneous saccharification and fermentation of sustainable agro-industrial residues

Wahib Al Abdullah [1], Yaser Dahman [1,*]

[1]Department of Chemical Engineering, Ryerson University, Toronto, Ontario CANADA M5B 2K3

*corresponding author e-mail address: ***ydahman@ryerson.ca***

ABSTRACT

The present study evaluates possibility to produce biocellulose nanofibers (BC) in simultaneous saccharification and fermentation (SSF). Pre-treated wheat straws (WS) were further incubated in the fermentation broth in the presence of *Gluconacetobacter xylinus* bacterium in the presence and absence of hydrolysis enzymes. WS were not filtered as common during separate hydrolysis and fermentation (SHF). Generally, results in the present study demonstrate that BC production in SSF is rather challenging, especially in the presence of hydrolysis enzymes. Total sugars produced during SSF were higher than SHF, and were generally identical under different pre-treatment and hydrolysis conditions (~54 g/L). This represents maximum amounts at complete hydrolysis of biomass due to the longer incubation time compared to SHF. Maximum BC production of 10.8 g/L was achieved when WS was chemically pretreated with 1% (by volume) dilute acid for 30 minutes at 121°C. Sample pre-treated with 2% acid at similar conditions resulted in 8.93 g/L BC produced. Typically, increasing duration and temperature of thermal treatment produced slightly more sugars, however, resulted in inhibited bacterial cells growth and resulted in slightly lower BC production. Considering that BC is also a good substrate for the *cellulase*, explains the higher concentration of remaining sugars (i.e., 15.50 g/L) when enzymatic hydrolysis was used. This led to lower yield of the final BC produced.

Keywords: *Biocellulose Nanofibers; Simultaneous Saccharification and Fermentation; Agro-Industrial Residues; Renewable Resources.*

1. INTRODUCTION

Cellulose is one of the most abundant components of biomass which is traditionally extracted from plant tissues (trees, cotton, etc.) [1]. Pure form of cellulose attains extraordinary biological and physical properties that are especially attractive in advanced applications in our everyday life [2-4]. The disadvantage of cellulose extracted from plant tissues is its contamination with organic impurities (hemicellulose and lignin). The purification process of these impurities requires insensitive chemical treatment that changes the polymer structure and significantly impacts its advanced characteristics [5, 6]. Cellulose can also be produced by certain bacterial species in fermentation yielding a very pure cellulose product with unique properties called Biocellulose Nanofibers (BC) [6, 7]. Microbial biocellulose (BC) is a highly crystalline and mechanically stable nanopolymer, which has excellent potential as a material in many novel applications [3-5]. The high surface-to-volume ratio of BC nanofibers combined with their unique properties such as the higher capacity for water, the higher permeability to oxygen, poly functionality, hydrophilicity and biocompatibility makes it an important material for different green biomedical applications [7-9].

Conventional production methods focus on BC synthesis by *Acetobacter* bacterial strain fermentation in an aerobic static or agitated culture containing nitrogen and carbon sources [9, 10]. A wide variety of simple sugars had been investigated and confirmed as a suitable carbon source feedstock in the fermentation media, the most common being glucose, fructose, xylose and sucrose [11-13]. The high economical cost and low production rate of BC using this conventional method form the main barrier of an industrial scale production of BC worldwide [9]. Various attempts had been made to overcome these challenges by investigating alternative feedstock as a carbon source in the process. Fruit juice, molasses, mixtures of sugars and many others had been investigated to decrees the process economical cost and improve its production rate [14-19]. Nevertheless, with the repetitive world food price crisis in recent years it is highly controversial to use highly demanded agricultural corpse in industrial production of materials. To solve these challenges and reach an economically feasible BC production in industrial scale we need to utilize renewable feedstock resources that have the ability to develop higher production yield than currently reached, and overcome the use of expensive carbon source feedstock in the culture media [4, 20].

The utilization of agricultural wastes is increasingly forming a new trend in biomaterials production research. The row materials commonly referred to as cellulosic wastes are widely agreed on as cheap renewable and sustainable organic source for fermentation [21, 22]. Biofuels, such as biodiesel, bioethanol, and biobutanol represent an industry that utilizes cellulosic wastes as a carbon source in their fermentation production [23, 24]. In the same manner earlier studies reported successful BC production from cellulosic wastes, like the use of cotton fabrics waste, liquor pulping and rice bark [25-27]. Moreover, earlier studies showed that wheat straws (WS), a widely available agricultural waste, hold high potential as an effective and economical feedstock in fermentation reaction [28, 29]. In two of our recent studies, we investigated two successful methods of BC production by utilizing

WS feedstock. Dahman et al. (2010) demonstrated improved BC production through using a combination of sugars mixtures that resemble WS hydrolysates [14]. In a later study, production of BC was investigated through utilizing agriculture residues (i.e., WS) in separate hydrolysis fermentation (SHF) [30]. BC was produced using hydrolyzed WS, as widely available agricultural residues, followed by its separate fermentation by *Acetobacter xylinum* bacterium. Different hydrolysis methods were investigated and a production of ~10.6 g/L was achieved when utilizing enzymatic hydrolysis. They concluded that the use of dilute acid in the hydrolysis increased total sugars extraction by more than 65% compared to that of water hydrolysis, while less than 15% increase of total sugars was attained using acidic solution with higher concentration. Thermal treatment at more intense conditions increased furfural concentration, which resulted in bacterial cells growth inhibition and leads to lower BC yield.

Utilization of green biocellulose nanofibers is a promising improvement to the industrial applications and our daily life. Yet, the high production cost of this class of biopolymers is one of the major challenges to overcome before reaching this goal, along with low production rate and long processing time. We postulated that further development to produce BC in SSF can be marked as significant improvement in the production of these green nanofibers. However, it is noticeable that there have been no attempts in the literature for producing bacterial cellulose in SSF. Against this background, the present work demonstrates an attempt to utilize agro-industrial residues to produce BC in SSF. After the preliminary pre-treatment of WS, resulting hydrolysate solution was incubated with BC producing bacteria in the presence of suspended WS (never been filtered). It is anticipated that further soaking of the WS may result in higher sugar hydrolysate concentration and thus improves BC production. Different acidic and thermal pre-treatment conditions of WS are examined in the present work. Productions of BC are then examined. Fermentation experiments are conducted in the presence and absence of WS hydrolysis enzymes. Effect of fermentation inhibitors liberated from WS is also investigated in the present study. Results obtained in this present study can be utilized with further development to produce the green biocellulose nanofibers at larger scale through utilizing renewable and sustainable substrate such as WS feedstock in environmental friendly SSF.

2. EXPERIMENTAL SECTION

2.1. Materials.

The following chemicals were obtained from Sigma-Aldrich and used as received: Agar, Ammonium Sulfate $(NH_4)_2SO_4$, *L(+)*Arbinose, D-Biotin, Calcium Carbonate ($CaCO_3$), Calcium Chloride Dihydrate ($CaCl_2.2H_2O$), Copper Sulfate Pentahydrate ($CuSO_4.5H_2O$), Ferrous Sulfate Heptahydrate ($FeSO_4.7H_2O$), Folic Acid, Fructose, *D-(+)*Galactose, Glucose, Hydroxymethylfurfural (HMF), Inositol, Magnesium Sulfate Heptahydrate ($MgSO_4.7H_2O$), Manganese Sulfate Pentahydrate ($MnSO_4.5H_2O$), Monopotassium Phosphate (KH_2PO_4), Nicotinic Acid, D-Pantothenic Acid, Pyridoxine Hydrochloride, Riboflavin, Sodium Molybdenum Oxide Dihydrate ($NaMoO_4.2H_2O$), Zinc Sulfate Heptahydrate ($ZnSO_4.7H_2O$), Thiamine Hydrochloride, and *D-(+)*-Xylose. Wheat Straw was collected from a local farm in Barrie, Ontario, and Corn steep liquor (CSL) was provided by Casco, London, ON, Canada, upon request, and used as received. *Gluconoacetobacter xylinum* (ATCC 700178) was supplied by American Type Culture Collection (ATCC), Manassas, VA 20108, USA.

2.2. Methods.

2.2.1. Pre-treatment and Hydrolysis of WS.

Physical pre-treatment were initially applied to all samples of WS, as all were grounded to fine particles using a hammer mill (Retsch GmbH Inc. USA) and filtered by 1.0 mm pore size sieve screen. Following that, a set of six different samples with each contains 20g of WS were pre-treated at different chemical and thermal conditions. Chemical pre-treatment was conducted using dilute sulphuric acid at different ratios of 1, and 2% by volume. Furthermore, two different temperature levels of 121 and 135°C and two different heating times of 30 and 90 minutes were examined for the thermal pre-treatment of WS. Following that, solutions pH were adjusted to 5 using 1 N NaOH solution prior to the incubation with the BC producing bacteria. Table 1 summarizes the different fermentations samples and their pre-treatment conditions.

Sample	Pretreatment Conditions: Acidic Treatment [a]	Pretreatment Conditions: Thermal Treatment	Enzymatic Treatment [b]
S1	1% H_2SO_4	121°C for 30 minutes	-
S2	2% H_2SO_4	121°C for 30 minutes	-
S3	1% H_2SO_4	121°C for 30 minutes	*Cellulase, β-glucosidase and Xylanase*
S4	1% H_2SO_4	121°C for 30 minutes	*Cellulase and β-glucosidase*
S5	1% H_2SO_4	121°C for 90 minutes	-
S6	1% H_2SO_4	135°C for 30 minutes	-

Table 1. Pre-treatment conditions of the different WS samples that were utilized in the production fermentation of BC.
[a] Total volume of 250 mL of dilute sulphuric acid solution
[b] Conducted with SSF at 29°C and initial pH of 5.0 for seven days by adding 0.375 mL from each of the enzymes

As shown in Table 1, the effect of acidic solution concentration was examined by samples S2 and S1. The effects of heating time and temperature were examined by samples S5 and S6. Moreover, samples S3 and S4 were chemically pretreated with dilute acid (i.e., 1% by volume), and then were subjected to enzymatic hydrolyses. Enzymatic hydrolyses were conducted

simultaneously with BC production in SSF at 29°C and initial pH 5.0. Sample S3 in Table 1 was hydrolyzed by adding 0.375 mL of each of the three enzymes *Cellulase, β-glucosidase*, *Xylanase*, while sample S4 was hydrolyzed by adding 0.375 mL of the two enzymes *Cellulase* and *β-glucosidase*.

2.2.2. Bacterial Strain and Culture Growth Conditions.

Gluconoacetobacter xylinus bacterium (ATCC 700178) was activated, in accordance with ATCC guidelines, using 50 g/L glucose, 5 g/L yeast, 12.5 g/L $CaCO_3$, and 15 g/L of agar were added with solid mediums. Liquid culture was prepared by transferring dry bacterial powder into sterile YGC 459 medium, and statically incubated (Symphony 8.5A, VWR) at 29°C and initial pH 5.0 (Easy Seven, Mettler Toledo) for 3 days. Bacterium cultivation on Agar plates was done by transferring liquid culture aseptically into Petri plates, containing YGC 459 Agar medium, and incubated at 29°C and initial pH 5.0 for 7 days. Inoculums solution was prepared by aseptically flooding the 7 days old culture plates with 20 mL sterile distilled water and gently suspending the culture with a cell spreader. Then the resulted solution was transferred to sterile inoculum tubes and mixed thoroughly using a VWR Analogue Vortex Mixer.

2.2.3. BC Production Experiments.

BC production experiments were conducted under sterile conditions in 500 mL shake flasks each containing 200 mL of the fermentation medium. Fermentation medium composition was as follows: 20 g or WS suspension solution (Carbon source), 5 mL of CSL (nitrogen source), 1 g/L of KH_2PO_4, 0.25 g/L of $MgSO_4.7H_2O$, 3.3 g/L of $(NH_4)_2SO_4$, 3.6 mg/L of $FeSO_4.7H_2O$, 14.7 mg/L of $CaCl_2.2H_2O$, 2.42 mg/L of $NaMoO_4.2H_2O$, 1.73 mg/L of $ZnSO_4.7H_2O$, 1.39 mg/L of $MnSO_4.5H_2O$, 0.05 mg/L of $CuSO_4.5H_2O$, 2 mg/L of Inositol, 0.4 mg/L of Nicotinic Acid, 0.4 mg/L of Pyridoxine Hydrochloride, 0.2 mg/L of D-Pantothenic Acid 0.2 mg/L of Riboflavin, 0.2 g/L of Folic Acid, 0.2 μg/L of D-biotin and 0.4 g/L of Thiamine Hydrochloride [7, 30]. All glassware was sterilized in the autoclave (Sanyo MLS 3780) at 121°C for 10 min prior to use. Pre-treated WS suspended in their hydrolysis solutions at initial pH of 5.0 and their additives were sterilized separately from CSL. The separate sterilization prevents high temperature reaction of sugars and amino acids at which produce black nitrogen containing compounds that impede microorganisms' growth [31]. CSL was aseptically added to the pretreated WS solution. Then sterile distilled water was added to compensate for evaporated water during sterilization in the autoclave. After each solution was cooled down to room temperature, samples S3 and S4 in Table 1 were inoculated with enzymes as described above. Each flask was then aseptically inoculated with 2 mL of the inoculums. All flasks were then incubated at 29°C for 7 days with shaking speed of 250 rpm (MaxQ 2000). At the end of the 7 days, the pH of each flask was checked, and solutions were treated with excess 2 N NaOH at 100°C for 5 min for cell lysis. An equivalent set of flasks was prepared according to conditions in Table 1. This set of flask was incubated for 7 days without the addition of the bacterium inoculums for BC production (equivalent to BC fermentation production time) in order to quantify the total produced sugars during hydrolysis of WS.

In all experiments, samples of 2 mL were aseptically collected right after inoculation and periodically thereafter till the end of 7 days. All samples were collected in a biosafety hood that is directly before used cleaned with ethanol and subjected to UV sterilization for 15 min. These collected samples were stored at -80°C until analyzed. Experimental results reported here are the averages of three biological replicates for all fermentation experiments.

2.2.4. Analytical Techniques.

Experimental results reported in the present work are the averages of three biological replicates for all fermentation experiments. Sugars and inhibitor concentrations were measured using high performance liquid chromatography (HPLC-Perkin Elmer) equipped with a refractive index detector (2414, Waters) and 5 mM H_2SO_4. Two HPLC columns were used separately, Shodex KC811 for measuring sugars concentration and Shodex SP0810 for measuring inhibitors concentration. The samples were centrifuged at 15000 rpm for 15 min and double filtered through 0.2 μm PTFE- filters (Whatman, USA). Each sample was analyzed under a flow rate of 0.6 ml/min and constant pressure. Concentrations were obtained using previously constructed calibration curves from the areas under corresponding peaks.

Viable cell counting was determined using a hemocytometer (QiuJing XB-K-25) having 1/400 mm^2 unit area and 0.1 mm height. The counting was done under optical microscope (Zeiss Axio Observer A1) at 50X magnification. Samples were diluted 20 folds and stained with florescent dye (BacLight ™, Bacterial Viability and Counting Kit) to differentiate between viable and nonviable cells.

Final BC production was quantified gravimetrically. After cell lysis, the solution containing produced BC was centrifuged at 4000 rpm for 15 min. The extracted BC was repeatedly washed with distilled water and centrifuged four times. The volume of extracted BC was then raised to 50 mL by adding distilled water, and the solution homogenized in a grinder (Kenmore) for 15 sec. Subsequently, 1 mL of the sample was transferred to a previously weighed crucible and placed in an oven at 80°C for one day to dry. The crucible containing dried sample was then cooled to room temperature and weighed to quantify BC production in g/L.

3. RESULTS SECTION

Figure 1 summarizes final BC production and corresponding pH of culture media at the end of fermentation production experiments. According to Figure 1, final BC production was generally in the range of 7.27 to 10.82 g/L, while final pH of the different culture media was for the majority of samples around 3.0 (±0.1). According to this Figure, the highest BC production of 10.82 g/L was achieved with sample S1. This sample was subjected to acidic pre-treatment using 1% (v/v) acidic solution for 30 minutes at 121°C (see Table 1). According to Table 1, samples S2, S5 and S6 that were exposed to more intense conditions produced lower BC of 8.93, 8.76 and 8.18 g/L, respectively. The lowest BC production was obtained from samples S3 and S4 (i.e., 7.27 and 7.21 g/L, respectively). Interestingly, both samples were subjected to enzymatic

hydrolysis that was conducted simultaneously with fermentation production of BC in SSF (see Table 1).

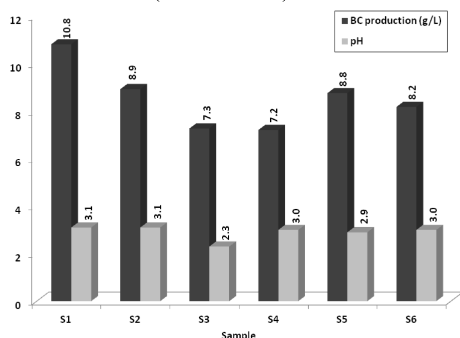

Figure 1. Results for the final BC production obtained using the different cultures prepared with conditions in Table 1, in addition to the final pH of the culture media.

According to Figure 1, final pH of all culture media for all samples dropped from the initial pH of 5.0 to mostly final pH in the range of 2.9 and 3.1. The decrease in final pH of all samples from the initial pH 5.0 in Figure 1 is due to the production of gluconic acid and/or acetic acid in the fermentation medium [31]. Usually, SSF demonstrates better pH stabilization ability in the fermentation medium that results in higher production yield of the targeted product [31]. However, a lowest pH of 2.3 was observed with sample S3. This sample was enzymatically pre-treated with three enzymes of *Cellulase, β-glucosidase, and Xylanase*. Enzymatic pre-treatment of sample S4 with two enzymes of *Cellulase* and *β-glucosidase* resulted in similar production of BC to S3 (i.e., ~7.2 g/L), but with final pH of 3.0. Apparently, using three enzymes during hydrolysis has boosted the cellular metabolism that led to higher production of acids. Similar results were obtained earlier by Wahib et al. [30].

It is generally known that enzymatic hydrolysis contributes in producing more sugars during hydrolysis step of agriculture residues [20]. Total amounts of sugars produced during each fermentation experiments and the total amounts of sugars consumed during fermentation experiments are listed in Table 2.

Table 2. Results obtained from the SSF experiments using WS as the feedstock to produce BC by *G. xylinus*.

Samples	BC Production (g/L)	Total Sugars (g/L)		Average cell concentration (10^7 cells/mL)	Average cell proliferation rate (10^4cells/mL.h)	BC Yield		Furfural (g/L)
		Available	Consumed			$Y_{P/C}$ [a]	$Y_{P/S}$ [b]	
S1	10.82	52.51	48.29	5.05	65.31	72.06	0.224	0.31
S2	8.93	52.87	44.33	3.94	50.34	57.95	0.201	1.21
S3	7.27	54.92 [c]	39.45	7.38	90.76	46.00	0.184	0.32
S4	7.21	54.04 [c]	40.56	6.40	88.99	39.67	0.177	0.31
S5	8.76	53.20	44.28	3.50	45.12	69.28	0.198	1.53
S6	8.18	53.52	44.63	3.27	42.00	54.48	0.184	1.82

[a] Calculated from the weight of final product/bacterial cell dry weight at the beginning of fermentation (not shown).
[b] Calculated from the weight of product/weight of total sugars consumed.
[c] Calculated from equivalent control hydrolysis experiment.

Results in Table 2 demonstrates approximately equivalent total sugars produced under different pre-treatment conditions. These closely equal values of produced sugars indicate that all samples reached a limit of maximum sugars extraction from the WS when incubated in the fermentation solutions during the 7 days. Further examination shows that thermal pre-treatment for longer time or at higher temperature (i.e., samples S5 and S6) resulted in slightly more total sugar production compared to samples with acidic treatment (i.e., samples S1 and S2). Sample S2 that was chemically pre-treated with higher acid concentration produced similar total sugars compared to sample S1. Moreover, samples subjected to enzymatic treatment (i.e., samples S3 and S4) resulted in producing the highest total sugars. It is expected that higher produced sugar leads to higher cellular growth activities. According to results in Table 2, the highest average cell concentrations and proliferation rates among all samples was achieved with samples S3 and S4. However, both of these samples achieved the lowest production of BC. Sample S1 with the highest BC production was accompanied with slightly lower total sugar production of 52.5 g/L and lower average cell concentration of 5.05×10^7 cells /mL (i.e., average cell proliferation rate of 65.31×10^4 cells /mL.h). Apparently, sugars produced by samples S3 and S4 improved mainly cells proliferations and maintenance, while sugars were utilized further towards higher BC production in sample S1. As listed in Table 2, total sugars that were consumed in sample S1 was the highest among all other samples (i.e., 48.29 g/L that represents 92%). Samples S3 and S4 that had the lowest production of BC consumed the lowest amounts of sugars (i.e., 39.45 and 40.56 g/L respectively, which are equivalent to 72 and 75%). The lower pH reached can impact the metabolism of the cells and lead to lower production as seen in Figure 1. Remaining samples recorded similar total sugars' consumption of ~83%, with approximately similar final BC production. The change in the percentage of remainder total sugar during fermentation time is summarized in Figure 2 during SSF production of BC. As shown in Figure 2, sugars were consumed at higher rates during the first 75 hours of fermentation for all culture media in Table 1. During this time period, SSF shows two different rates for the change in total sugars concentration percentages. Samples S1, S3 and S4 had higher total sugar consumptions, with ~50% of sugars being consumed during the first 50 hours of fermentation. This can be explained by the higher average cell concentration in Table 2 (thus higher average cell proliferation rates).

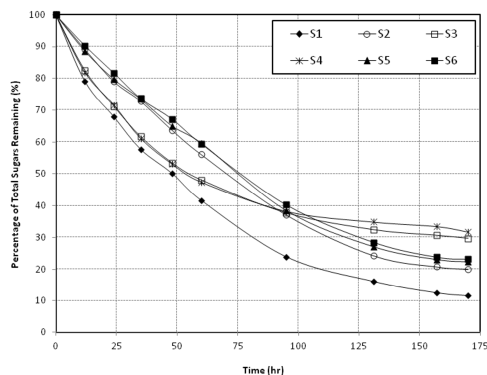

Figure 2. Percentage of remaining sugar concentration with time during the different SSF production experiments as listed in Table 1.

Moreover, samples S2, S5 and S6, which were subjected to intensive pre-treatment conditions and lower average cell concentration, recorded lower total sugar consumptions with ~35% of total sugars were consumed in the first 50 hours of SSF. Toward the end of the fermentation, sugars consumption rates reduced significantly for all samples and reached minimum at the end. Generally, samples S3 and S4 reached the end of fermentation with ~25-28% unconsumed sugars. It is worth assuming that produced BC can also be a good substrate for the enzymatic hydrolysis by *cellulase*. This may result in decreasing the yield of BC. This can also explain the higher concentration of the remaining sugars in Table 2 for samples S3 and S4. According to this, it is challenging to solve the problem since the goal of saccharification of cellulosic substrates is contrary to the goal of production of BC in SSF in the presence of hydrolysis enzymes. Samples S2, S5 and S6 ended the 7 days of SSF with similar amounts of unconsumed sugars (i.e., average of ~17%). Whereas, sample S1 recorded lowest unconsumed total sugars towards the end of the fermentation (i.e., ~8%). Generally, fermentation inhibition of the available sugars consumption can be related to the production of two fermentation inhibitors during the hydrolysis of WS (i.e., furfural and 5-hydroxymethyl-furfural (5-HMF)) [20, 30, 32]. In the present study, negligible amounts of 5-HMF were observed in all samples of WS hydrolysis. Results in Table 2 show that samples S1, S3 and S4 produced approximately 0.3 g/L of furfural. Moreover, samples S2, S5 and S6 produced higher furfural of 1.21, 1.53, and 1.82 g/L, respectively. It is important to understand that furfural is produced by dehydration of hemicelluloses when subjected to intensive heating in the presence of sulfuric acid [33]. This explains the increase in furfural concentration as acid concentration or thermal treatment temperature and time were increased during pre-treatment in Table 1. Results show that the higher furfural concentrations considerably impacted cells growth and proliferation for samples S2, S5, and S6 (Table 2). Similar inhibitory effects of furfural were reported in the literatures [30, 34]. Interestingly, total sugars consumptions for the three samples were approximately identical, and were not affected by the lower cell counts. The higher total sugars consumptions with lower cell proliferation rates that were obtained with samples S2, S5 and S6 led to higher production of BC compared to samples S3 and S4. Apparently, the higher furfural production that was observed with samples S2, S5, and S6 of 1.21, 1.53, and 1.82 g/L inhibited further utilization of total sugars during SSF that resulted in lower BC production compared to sample S1. Yields calculated for BC per gram of bacterial cell dry weight (Y_{PC}) and per total sugars consumed (Y_{PS}) showed maximum of both values for sample S1 (i.e., 72.06 and 0.22 g/g respectively). This is associated with the maximum production observed earlier. Samples that were hydrolyzed using enzymes showed low yields among other samples in Table 2. These lower yields are due to the lower BC productions that were associated with the lower sugar consumptions and higher cell concentrations. Samples that were hydrolyzed under more intense conditions in terms of acid concentration in addition to temperature of thermal treatment (i.e., samples S2 and S6) resulted in slightly lower yields compared to sample S1 due to the lower BC production. Sample S5 with more thermal duration showed higher yield than all samples in Table 2 except for Sample S1. Figure 3 shows concentrations of individual sugars that were produced during hydrolysis in addition to the corresponding final concentration of individual sugars that were not utilized towards BC production during the SSF. Results show that initial sugars content in all pre-treated WS samples contain ~53% glucose and ~30% xylose, while the remaining individual sugars in Figure 3 such as galactose, mannose and arabinose form ~16% of the total sugars produced. Fructose, which is well known to promote BC production, had negligible concentrations in all samples although it exists in the dry basis of WS [14, 30, 35]. This complies with previous literature that demonstrated the absence of fructose in pre-treated WS [36, 37]. WS is composed of 39% cellulose and 32% hemicelluloses on dry basis, which are the primary source of glucose and xylose after hydrolysis [20, 38, 39].

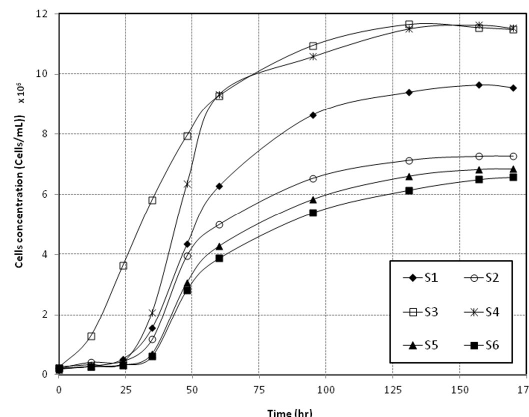

Figure 3. Estimated individual sugars' concentrations available for fermentation and final individual sugar concentration quantified at the end of the SSF experiments.

The high glucose concentrations in samples S3 and S4 compared to the rest of the samples comes from utilizing *Cellulase* and *β-glucosidase* enzymes during the hydrolysis step. Similarly, the high concentration of xylose in S3 is due to utilizing *Xylanase* enzyme for the hydrolysis of this sample. Further examination of results in Figure 3 shows that concentrations of glucose in the media at the end of fermentation were in the range of 4.2 to 7.6 g/L. Lowest glucose concentration was observed with sample S1 that consumed most of glucose during fermentation with 1.73 g/L remaining glucose (i.e., consumption of 93.42%; see Table 3). Moreover, individual sugars concentrations left at the end of fermentation of sample S1 recorded lower concentrations compared to the rest of the samples (i.e., 6.58-11.74% in Table 3).

Table 3. Percentages of individual sugar consumption during the production of BC in SSF with pre-treated WS in the feedstock.

Sample	Glucose	Galactose	Mannose	Xylose	Arabinose
S1	93.42	90.56	89.83	88.26	89.60
S2	86.40	85.59	86.07	82.09	86.64
S4	75.92	71.93	64.48	66.72	72.01
S5	83.34	83.62	85.59	82.49	85.62
S6	82.76	84.41	84.67	81.78	84.76

This justifies the maximum total sugar consumption that is reported in Table 2, which resulted in the highest BC production. Meanwhile, samples S3 and S4 with the lowest total sugars consumptions that led to the lowest BC production in Table 2 demonstrated lower consumptions of the individual sugars with glucose consumption being relatively the highest. Samples S2, S5, and S6 with almost similar BC production recorded approximately similar consumption of individual sugars in Table 3. Figure 4 illustrates the change in the viable bacterial cell concentrations during the course of SSF for the different samples in Table 1.

A delay in *G. Xylinus* growth was observed between ~30 to 35 hours for all samples except for sample S3. Following that, rapid exponential growth phase was observed over a period of ~25 hours. Cells concentration reached maximum concentration that settled constant to the end of fermentation. Maximum cell concentration of ~12×10^7 cells/mL was observed with samples S3 and S4. A lower cell concentration of ~9.5×10^7 cells/mL was observed with sample S1, while lowest cells concentrations in Figure 4 was observed with samples S2, S5 and S6 (i.e., ~6.5×10^7 - 7×10^7 cells/mL). This rapid growth of cells concentration in S3, with shorter delay phase, is related to the high xylose concentration obtained (see Figure 3). This was achieved due to the enzymatic pre-treatment with *Xylanase* that breaks hemicellulose to the xylose sugar [20]. Xylose is metabolized by *G. Xylinus* for bacterial cells proliferation and its oxidation produces acetic acid that reduces the medium pH [14, 30]. This explains the pH drop in sample S3 in Figure 1 to a lower value than the rest of the samples at the end of fermentation. Samples S2, S5 and S6 apparently showed that the high furfural concentration in Table 2 considerably affected cells growth and proliferation. This resulted in lower sugar consumption, and as a result in lower BC production compared to sample S1. Similar effect of furfural on cell growth has been reported earlier in the literatures [40].

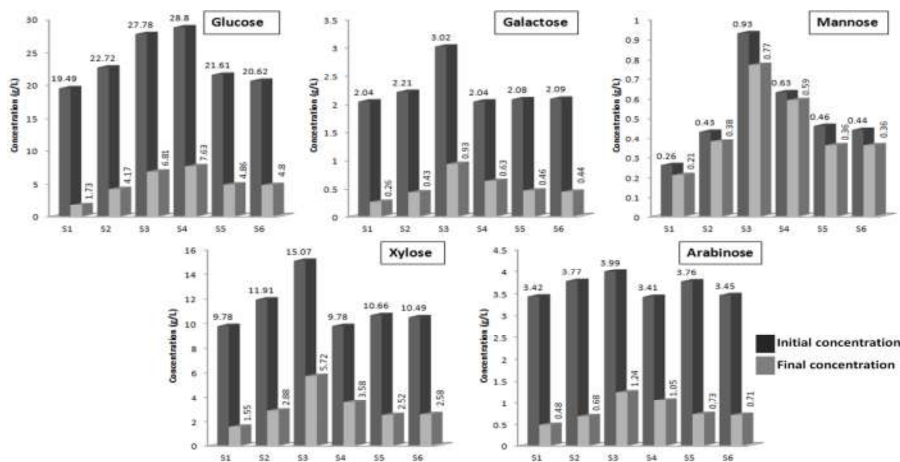

Figure 4. Changes in G. Xylinus cell counts obtained during different SSF experiments as listed in Table 1.

4. CONCLUSIONS

This study presents results for the production of BC in SSF. Results showed that maximum BC production of ~10.8 g/L was obtained with WS simple pretreatment using 1% dilute acid at 121°C for 30 minutes. Total sugars produced in the present study were approximately similar with slight different for the different pre-treatments employed. Apparently the longer time of incubating the WS during the SSF allowed for maximum release of hydrolyses sugars. Thermal pre-treatment for longer time or at higher temperature resulted in slightly more total sugar production compared to samples with acidic treatment. Pre-treatment with higher acid concentration produced similar total sugars. Apparently, the increase in the concentration of furfural produced during pre-treatment under more intense conditions of acid concentrations and thermal pre-treatment temperature has resulted in lower BC production. Enzymatic hydrolyses resulted in higher total hydrolysate sugars produced in addition to the highest average cell concentrations and proliferation rates. Final BC production was proportional to the total consumption of sugars with simple acidic hydrolysis being the highest in both quantities. The higher shift in fermentation media pH observed with enzymatically hydrolyzed samples impacted the metabolism of the cells and led to lower sugar consumption and lower BC production. Results obtained from the individual sugars production demonstrated rapid growth of cells associated with higher xylose concentration available. This was obtained due to the utilization of enzymatic pre-treatment with *Xylanase*. Oxidation of xylose resulted in the highest shift in fermentation media pH (i.e., pH of 2.3 compared to an initial value of 5.0).

5. REFERENCES

[1] de Souza Lima M.M., Borsali R., Rodlike cellulose microcrystals: structure, properties, and applications, *Macromol. Rapid Comm.*, 25, 771–787, **2004.**
[2] Khan F., Dahman Y., Novel Approach for the Utilization of Biocellulose Nanofibres in Polyurethane Nanocomposites for Potential Applications in Bone Tissue Implants, *Des. Monomers. Polyml.*, 15, 1-29, **2012.**
[3] Sani A., Dahman Y., Improvements in the Production of Bacterial Synthesized Biocellulose Nanofibers Using Different Culture Methods, *J. Chem. Technol. Biotechnol.*, 85, 151–164, **2010.**
[4] Shoda M., Sugano Y., Recent advances in bacterial cellulose production, Biotechnol. *Bioprocess. Eng.*, 10, 1–8, **2005.**
[5] Dahman Y., Nanostructured Biomaterials and Biocomposites from Bacterial Cellulose Nanofibers, *J. Nanosci. Nanotechnol.*, 9, 5105–5122, **2009.**
[6] Fontana J.D., de Souza A.M., Fontana C.K., Torriani I.L., Moreschi J.C., Gallotti B.J., de Souza S.J., Narcisco G.P., Bichara J.A., Farah L.F., Acetobacter cellulose pellicle as a temporary skin substitute, *Appl. Biochem. Biotechnol.*, 25, 253–264, **1990.**
[7] Iguchi M., Yamanaka S., Budhiono A., Bacterial cellulose – a masterpiece of nature's arts, *J. Mater. Sci.*, 35, 261–270, **2000.**
[8] Hench L.L., Biomaterials: a forecast for the future, *Biomaterials*, 19, 1419–1423, **1998.**
[9] Geyer U., Heinze T.H., Stein A., Klemm D., Formation, derivatization and applications of bacterial cellulose, *Int. J. Biol. Macromol*, 16, 343–347, **1994.**
[10] Chao Y., Ishida T., Sugano Y., Shoda M., Bacterial cellulose production by Acetobacter xylinum in a 50-L internal-loop airlift reactor, *Biotechnol. Bioeng.*, 68, 345–352, **2000.**
[11] Dahman Y., Production and Utilization of Bacterial Cellulose Nanofibres, *Encyclopedia of Nanoscience and Nanotechnology*, 21, 459-479, **2009.**
[12] Colvin J.R., Leppard G.G., The biosynthesis of cellulose by Acetobacter xylinum and Acetobacter acetigenus, *Can J Microbiol.*, 23, 701–709, **1977.**
[13] Mikkelsen D., Flanagan B.M., Dykes G.A., Gidley M.J., Influence of different carbon sources on bacterial cellulose production by Gluconacetobacter xylinus strain ATCC 53524, *J. Appl. Microbiol.*, 107, 576-583, **2009.**
[14] Dahman Y., Jayasuriya K.E., Kalis M., Potential of Biocellulose Nanofibers Production from Agricultural Renewable Resources: Preliminary Study, *Appl. Biochem. Biotechnol.*, 162, 1647–1659, **2010.**
[15] Kurosumi A., Sasaki C., Yamashita Y., Nakamura Y., Utilization of various fruit juices as carbon source for production of bacterial cellulose by Acetobacter xylinum NBRC 13693, *Carbohyd. Polym.*, 76, 333–335, **2009.**
[16] Hong F., Qiu K.Y., An alternative carbon source from konjac powder for enhancing production of bacterial cellulose in static cultures by a model strain Acetobacter acetisubsp. xylinus ATCC 23770, *Carbohydr. Polym.*, 72, 545-549, **2008.**
[17] Bae S., Shoda M., Bacterial cellulose production by fed-batch fermentation in molasses medium, *Biotechnol. Prog.*, 20, 1366–1371, **2004.**
[18] Noro N., Sugano Y., Shoda M., Utilization of the buffering capacity of corn steep liquor in bacterial cellulose production by Acetobacter xylinum, *Appl. Microbiol. Biotechnol.*, 64, 199-205, **2004.**
[19] Jung H.I., Jeong J.H., Lee O.M., Park G.T., Kim K.K., Park H.C., Lee S.M., Kim Y.G., Son H.J., Influence of glycerol on production and structural-physical properties of cellulose from Acetobacter sp. V6 cultured in shake flasks, *Bioresour. Technol.,* 101, 3602–3608, **2010.**
[20] Thirmal C., Dahman Y., Comparisons of Existing Pretreatment, Saccharification, and Fermentation Processes for Butanol Production from Agricultural Residues, *Can. J. Chem. Eng.*, 90, 745–761, **2012.**
[21] Kumar P., Barrett D.M., Delwiche M.J., Stroeve P., Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production, *Ind. Eng. Chem. Res.*, 48, 3713–3729, **2009.**
[22] Rubin E.M., Genomics of cellulosic biofuels, *Nature*, 454, 841-845, **2008.**
[23] Qureshi N., Sahaa B.C., Hectora R.E., Hughesb S.R., Cottaa M.A., Butanol production from wheat straw by simultaneous saccharification and fermentation using Clostridium beijerinckii: part I – batch fermentation, *Biomass Bioenerg*, 32, 168–175, **2008.**
[24] Ezeji T.C., Qureshi N., Blaschek H.P., Bioproduction of butanol from biomass: from genes to bioreactors, *Curr. Opin. Biotech*, 18, 220–227, **2007.**
[25] Uraki Y., Morito M., Kishimoto T., Sano Y., Bacterial cellulose production using monosaccharides derived from hemicelluloses in water-soluble fraction of waste liquor from atmospheric acetic acid pulping, *Holzforschung*, 56, 341–347, **2002.**
[26] Goelzer F.D.E., Faria-Tischer P.C.S., Vitorino J.C., Sierakowski M.R., Tischer C.A., *Mat. Sci. Eng. C.*, 29, 546-551, **2009.**
[27] Kuo C.H., Lin P.J., Lee C.K., Enzymatic saccharification of dissolution pretreated waste cellulosic fabrics for bacterial cellulose production by Gluconacetobacter xylinus, *J. Chem. Technol. Biot.*, 85, 1346–1352, **2010.**
[28] Hong F., Zhu Y.X., Yang G., Yang X.X., (2011) Wheat straw acid hydrolysate as a potential cost-effective feedstock for production of bacterial cellulose, *J. Chem. Technol. Biot.*, 86, 675–680, **2011.**
[29] Volynets B., Dahman Y., Assessment of Pretreatments and Enzymatic Hydrolysis of Wheat Straw as a Sugar Source for Bioprocess Industry, *I.J.E.E.*, 2, 427-446, **2011.**
[30] Al-Abdallah W., Dahman Y., Production of green biocellulose nanofibers by Gluconacetobacter xylinus through utilizing the renewable resources of agriculture residues, *J. Chem. Technol. Biot.*, 36, 1735-1743, **2013.**
[31] Yang Y.K., Park S.H., Hwang J.W., Pyun Y.R., Kim Y.S., Cellulose production by Acetobacter xylinum BRC5 under agitated condition, *J. Ferment. Bioeng.*, 85, 312–317, **1998.**
[32] Modig T., Liden G., Taherzadeh M.J., The kinetics of furfural inhibition of the enzymes alcohol dehydrogenase, *Biochem. J.*, 363, 769–776, **2002.**
[33] Singh A., Das K., Sharma D.K., Production of xylose, furfural, fermentable sugars and ethanol from agricultural residues, *J. Chem. Technol. Biot.*, 34, 3451–3461, **1984.**
[34] Dahman Y., Ugwu C., Production of green biodegradable plastics of poly(3-hydroxybutyrate) from renewable resources of agricultural residues, *Bioproc. Biosyst. Eng.*, 37, 1561-8, **2014.**
[35] Brown R.M., Cellulose Structure and Biosynthesis- What's in Store for the 21st Century, *J. Polym. Sci. A: Polym. Chem.*, 42, 487–495, **2004.**
[36] Duarte L.C., Silva-Fernandes T., Carvalheiro F., Girio F.M., Dilute acid hydrolysis of wheat straw oligosaccharides, *Appl. Biochem. Biotech.*, 153, 116–126, **2009.**
[37] González G., López-Santín J., Caminal G., Solà C., Dilute acid hydrolysis of wheat straw hemicellulose at moderate temperature: a simplified kinetic model, *Biotech. Bioeng.*, 28, 288–293, **1986.**
[38] Zabihi S., Alinia R., Esmaeilzadeh F., Kalajahi J.F., (2010) Pretreatment of wheat straw using steam, steam/acetic acid and steam/ethanol and its enzymatic hydrolysis for sugar production, *Biosys. Eng.*, 105, 288-297, **2010.**
[39] Sirisansaneeyakul S., Rizzi M., Hydrolysis of wheat straw hemicelluloses, *Kasetsart J. (Nat. Sci.)*, 32, 224-233, **1998.**
[40] Peng L.U., Chen L.J., Lia G.X., Shen S.H., Wang L.L., Jiang Q.Y., Zhang J.F., Influence of Furfural Concentration on Growth and Ethanol Yield of Saccharomyces Kluyveri, *J. Environ. Sci.*, 19, 1528–1532, **2007.**

6. ACKNOWLEDGEMENTS

Authors would like to acknowledge financial support from Agriculture and Agri-Food Canada, the Natural Sciences and Engineering Research Council of Canada (NSERC), and the Faculty of Engineering and Architectural Science at Ryerson University in Toronto, Canada.

© 2016 by the authors. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution license (http://creativecommons.org/licenses/by/4.0/).