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Production of Green Biocellulose Nanofibers Through Utilizing Agricultural Wastes

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PRODUCTION OF GREEN BIOCELLULOSE NANOFIBERS THROUGH UTILIZING AGRICULTURAL WASTES

by

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Wahib AL-Abdallah

ABSTRACT

Biocellulose Green Nanofibers production form Agricultural Wastes

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In the present study, the green Biocellulose Nanofibers (BC), a vitally emerging biomaterial, was produced by fermentation of wheat straw (WS), as a widely available agricultural waste, using. Two different fermentation methods were used; Separate Hydrolysis and Fermentation (SHF), and Simultaneous Saccharification and Fermentation (SSF). Different acidic and enzymatic WS pretreatment conditions were used to understand the effect of pretreatment conditions on BC production. Afterward, sugar hydrolsates were simultaneously or separately inoculated with *Gluconacetobacter Xylinum* bacterium (i.e., for SSF and SHF, respectively), at optimum production conditions in shake flasks for 7 days to produce the biocellulose nanofibers. BC productions of 9.7 g/L in SHF and 10.8 g/L in SSF were achieved when WS was pretreated with dilute acids. Enzymatic treatment of WS after acidic pretreatment increased sugars' concentrations from the hydrolysis, which increased BC production in SHF to 10.6 g/L. However, enzymes in SSF broke cellulose I alpha linkage in BC and decreased its production compared to no enzymatic treatment. Results show that glucose extracted from WS (~55% of total sugars) was found essential for the cellular metabolism, while xylose (~28% of total sugars) was highly consumed during cells growth phase. Generally, increasing thermal treatment, time and temperature, resulted in increasing furfural concentration. This observed to inhibits bacterial cells growth and leads to lower nanofibers yield when exists at concentration higher than 1 g/L threshold.

In general, results obtained in the present study demonstrate the ability of utilizing agricultural wastes in the fermentation production of BC. Such a step is expected to eliminate cost of expensive pure sugars as a carbon source in the fermentation. Also the study shows an improved production yield by using effective fermentation techniques as SSF compared to classical methods used in literature.

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DEDICATION

To she who burns her fingers to light our way

…. To my mother

TABLE OF CONTENT

NOMENCLATURE

Symbol	Description
BC	Biocellulose nanofibers, Bacterial cellulose
CSL	Corn steep liquor, traditionally used as a nitrogen source in fermentation
F1	SHF control sample acidic pretreatment
F2	SHF high acid concentration sample
F3	SHF soaking of WS for 3 hours sample
F4	SHF WS treatment with 3 enzymes sample
F5	SHF WS treatment with 2 enzymes sample
F6	SHF acidic pretreatment longer thermal treatment time
F7	SHF acidic pretreatment higher thermal treatment temperature
F8	SHF water pretreatment reference sample
F9	SHF water pretreatment longer time
F10	SHF water pretreatment higher temperature
G. Xylinus	Gluconoacetobacter Xylinum (ATCC 700178)
HPLC	High performance liquid chromatography
MO	Molasse, traditionally used as a carbon source in fermentation
S1	SSF control sample acidic pretreatment
S2	SSF high acid concentration sample
S3	SSF WS treatment with 3 enzymes sample
S4	SSF WS treatment with 2 enzymes sample
S5	SSF acidic sample longer thermal treatment time
S6	SSF acidic pretreatment higher thermal treatment temperature
SHF	Separate hydrolysis fermentation
SSF	Simultaneous scarification fermentation
STDEV	Standard deviation of data points

WS	Wheat Straw
µV.s	Micro volt seconds (HPLC graph area having µV verses seconds plot)
%RSD	Percent relative standard deviation of data points

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1. INTRODUCTION

The majority of commodities in our everyday life are based of non-renewable resources such as minerals, petrochemicals, and natural gas. Unfortunately, the utilization of these products is associated with many environmental, social and economical problems, such as environmental degradation, processing health hazards, increasing costs, and depleting recourses. In the efforts to solve these challenges, the industrial sectors are increasingly focussing on developing processes that utilize renewable resources and provide competitive products to satisfy the market needs. The most prominent topic in this trend is “biomaterials” production. Where, biomaterials are based on living organic systems, such as agricultural crops, and provide various interesting benefits, such as environmental friendliness, low economical costs, and promising advanced properties [1].

Cellulose is one of the important clusters of biomaterials due to its unique physical and chemical properties. It is composed of glucose monomers connected by β (1-4) glycosidic linkages, with chemical formula $(C_6H_{10}O_5)_n$. The structure of pure cellulose has a native dimension of less than 50 nm in diameter which makes it a natural nanofiber. Also, cellulose fibers have ultimate breaking strengths up to 10,000 MPa, which is 2.5 time that of structural steel and close to that of carbon fiber. Its polymer is of hydrophilic nature, and has a high aspect ratio with a fiber diameter of 20-100 nm. All these properties gave pure cellulose its high optical transparency, very high surface area per unit mass and very high liquid loading capacity. Which all make it highly suitable for numerous advanced applications [2]. In nature, cellulose forms the basic structural matrix of the cell walls in all plants. Cellulose is the most abundant organic material on earth and the most renewable substance at the same time. More than

100 billion tones of cellulose are biosynthesized annually, as its content in various materials ranges as 98% high in cottons, to 60-75% in plant fibers, and 40-50% in wood [3]. Plant cellulose is most commonly used in pulp and textile industries. However, plant cellulose is not pure and contains many contaminations, including hemicellulose and lignin, which requires harsh chemical treatments to remove most of the impurities. The chemical purification of plant cellulose result in irreversible alterations of its structure, which eventually deprive the polymer of its advanced characteristics, and negatively impacting its functionality in advanced applications [4].

Meanwhile, biocellulose (BC) is a 100% pure form of cellulose nanofibers that consists of higher structural crystallinity and higher degree of polymerization [5,6]. This exceptional pure structure helps BC attain the unique physical and biological properties, such as ductility, high tensile strength, oxygen permeability and biocompatibility, in addition to the properties described before [7]. All those superior properties of BC make it a highly potential precursor for breakthrough technologies in many vital fields, such as membrane technologies, green biotechnology, and hybrid nanocomposites [2,8]. The application side of such developments would lead to cutting edge products, like artificial skin, blood vessel substitute, bone scaffold, and electromagnetic papers [4,6]. Such future advancements will bring tremendous social and economical benefits to human kind [2]. Moreover, the high scale production of BC would favour it as a better substitute to plant cellulose in a wide range of application in our everyday life [9].

Synthesis of BC is accomplished by Acetobacter bacterial species in the presence of a carbon and nitrogen sources in the fermentation medium [5,10]. Conventional methods of BC production utilize various types of simple sugars as a carbon source. Fructose, glucose, sucrose and xylose have been repeatedly used in the production of BC by *G. Xylinus* bacteria in both

static and agitated cultures, and in various different reactor configurations [10-12]. The hurdle in scaling up BC production using these feedstocks rest in their high economical cost and relatively low production yield [8]. In the recent years, several attempts with alternative feed stocks have been tested to improve the production yield of BC and decrease the economical cost. This includes examining production through utilization of sugar mixtures, fruit juices, konjak, molasses, corn steep liquor and organic acids [13-18]. However, the use of agricultural crops in large scale consumptive industries is highly debatable as it can affect the safety of animal and human food chain, and the ecological balance in our nature. This creates a bottleneck in advancing BC production that necessitate the examination of utilizing renewable feedstock resources that resolve the high economic cost of the culture medium, and its associated social and ecological impacts [8,19]. This is given the objective of continuing the improvement of production and yield of the green product.

Generally, a recent trend in research has been to produce green products through utilizing the sustainable and renewable resources of agricultural wastes, commonly referred as cellulosic wastes. Most popular attempts are towards the production of green biofuels such as bioethanol and biodiesel [20,21]. A variety of agricultural wastes, including wheat straw (WS), rice straw, switch grass and corn fiber had been successfully utilized in the production of second generation biofuels [22,23]. Further green chemicals and biomaterials have been investigated all based on the utilization of agricultural wastes such as biosorbents to sequester chemicals of interest, or water treatment by fungal culture [24,25].

Considering this background, the main objective of the present study is to produce the biocellulose nanofibers using cheaper and renewable resources of agriculture wastes. Interestingly, several attempts were made to produce BC from cellulosic wastes, like the use of

liquor pulping, rice bark and cotton fabrics waste, but, still the production yield lacks major improvements [26-28]. At the same time the preliminary studies on WS hydrolysates fermentation by *G. Xylinus* shows that WS holds a high potential in improving the production of BC supported by its vast renewability, and the high sugar concentration in its biomass [13,29,30]. All these make BC production from WS a promising alternative to investigate.

In the present work, production of the biocellulose nanofibers was examined through two methods, a separate hydrolysis and fermentation process (SHF), and a simultaneous scarification fermentation process (SSF). Initially, WS was pretreated, and then produced sugars were utilized as the feedstock to the BC producing fermentation. Different pretreatment conditions of WS were examined that includes dilute acids of different strengths, heating time and temperatures, and using enzymes. Effect of inhibitors produced during fermentation will also be investigated. It is strongly believed that results from this study will facilitate the long-term goal to produce novel nanofibers at lowest cost possible. This would help to bring several biocellulose nanofibers based products to the local and international market at reasonable prices considering their properties.

2. LITREATURE REVIEW

1.1 Investigation of Cellulose nanofibers

2.1.1 Structure of Cellulose

Cellulose is a linear polymer consisting of repeated glucose monomers as shown in Figure 1. In plant cells glucose particles are polymerized with β (1-4) linkage to form a linear polysaccharide. This process is catalyzed with the enzyme *Cellulose Synthase*. The produced polymer starts to accumulate outside the cell and form the cell wall (Figure 2).

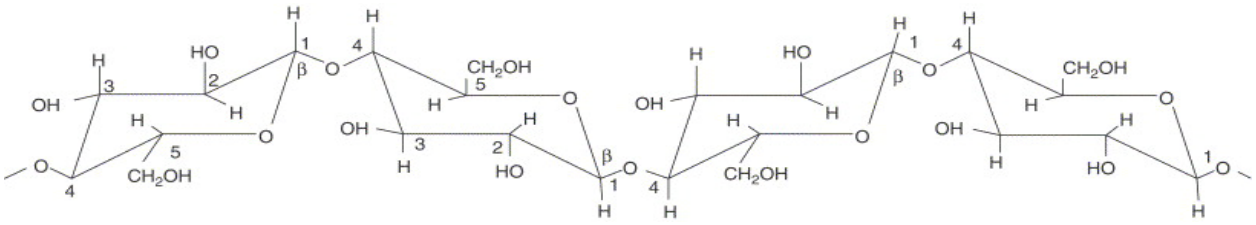

Figure 1. The structural formula of cellulose [31]

A hydrogen bonding connects the glucan chains with each other. The joint between these glucan chains start to form a microfibril, which is considered the basic structural unit of cellulose. The microfibrils are then connected into bundles, and later into ribbons, which leads to crystallization of the polymer, (Figure 3) [32]. The structural function of cellulose in plants is to protect the cells. It forms the skeleton of cell walls and responsible for keeping and providing its shape under tough conditions. In addition, it forms a driving force for the plant growth by controlling the size and direction of its elongation.

The dimensions of cellulose of ribbons vary depending on the study source. According to Brown et al. (1976) it is 3.2 nm (thickness) x 133 nm (length) [33], while Zaar et al. (1977) mentioned its dimensions as 3-4 x 70-80 nm [34], and according to Yamanaka et al. (2000) it is 4.1 x 117 nm [35]. The differences in ribbons dimensions may be reasoned to the differences in production processes over time.

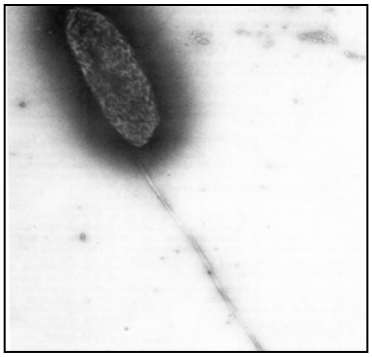

Figure 2. A ribbon of cellulose microfibril outside the cell [36]

The number of microfibrils in a cellulose pallet is controlled by the type of enzyme responsible for the synthesis of cellulose in the plant. Also, the glucan chains structures come in various sizes and network arrangements. For example, the large square structures with almost 1200 microfibrils in *Valonia*, the thin membrane-like structures in *Erythrocladia*, or the rectangular shaped with low number of glucan chains in *Boergesenia forbsei.*

Cellulose in general occurs in two forms with different degrees of crystallization, they are commonly referred to as cellulose I and cellulose II [32]. Where cellulose I is the most popular form, in which the glucan chains are parallel and arranged into microfibrils. It consists of two sub structures known as cellulose I_α and cellulose I_β. Cellulose I_α has a single triclinic unit molecule in its chain, where cellulose I_β has two monoclinic unit molecules in its chain and had

higher degree of crystallinity, however it is less met in nature as a pure form. As for cellulose II, they have anti-parallel glucan chains, and have an additional hydrogen bond per glucose triclinic molecule. This makes cellulose II a stronger and more stable form than that of cellulose I. Yet, cellulose II is produced in minor quantities by only few kinds of bacteria and some algae.

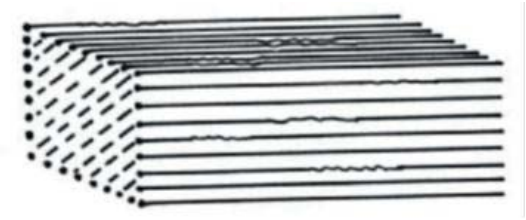

Figure 3. A schematic diagram in a single glucan chain polymer met in the cellulose microfibrils [32]

2.1.2 Cellulose producers

The majority of cellulose used in the industry comes from trees and cotton plants. In addition, a wide variety of living organisms is capable to produce cellulose. Higher plants like corn roots, mung bean hypocotyls, radish roots, and coleoptiles are well known to produce cellulose. Moreover, land plants such as mosses, ameobae, ferns, certain fungi (the *Oomycetes*), angiosperms and gymnosperms, cellular slime molds (*Dictyostelium discoideum*) and a great diversity of algae (*Vaucheria Glaucocystis*, *Pleurochrysis*, *Oocystis*, *Valonia*, *and Eremosphaera*), plankton and marine algae all have the ability to produce cellulose [37]. In animals cellulose is also produced by a submarine animal called Urochordates living in the sea waters. Furthermore, X-ray diffraction testing on human patients of the disease S*cleroderma* reported elevated levels of cellulose, which appears as a hardening of skin or other organs [37,38].

Table 1. An overview of cellulose synthesizing bacteria's [39]

Genus	Cellulose structure
Acetobacter	Extracellular pellicle composed of ribbons
Achromobacter	Fibrils
Aerobacter	Fibrils
Agrobacterium	Short fibrils
Alcaligenes	Fibrils
Pseudomonas	No distinct fibrils
Rhizobium	Short fibrils
Sarcina	Amorphous cellulose
Zoogloea	Not well defined

Several bacterial genera are reported to synthesize biocellulose nanofibers. Among these are *Acetobacter*, *Rhizobium* (responsible for nodules in plants roots and assists in nitrogen fixation), and *Agrobacterium* (responsible for crown gall disease in plants). A summary of the most common BC producers is given in Table 1 [39].

2.1.3 *Xylinum* species

Xylinum is the most agreed on BC producer in terms of efficiency and the most studied among the genus in Table 1. Some of its common called names are *Acetobacter Xylinum, A. Xylinus, A.aceti* ssp., and *Xylinum*. Although, it was reclassified in 1998 and added to novel genus *Gluconacetobacter*, as *G. Xylinus* [40]. It was used by A.J. Brown in 1886 in the earliest studies that described biocellulose. It was until 1954 when Hestrin et al. (1954) opened the door of BC investigation after finding that *Xylinum* cells are capable of producing cellulose in the presence of glucose in the medium [37].

Xylinum is a gram negative strain which make it functional under obligate aerobic conditions. *Acetobacter* produces ribbons of cellulose from the surface of bacteria directly into the fermentation medium, and form a BC thin film. In static culture, the BC microfibers reach the surface and create a thin hydrophilic membrane commonly referred as a pellicle. While, other BC producing strains like *Alcaligenes, Agrobacterium* and *Rhizobium* synthesize only a bundle of few microfibrils.

2.1.4 Characteristics and applications

BC has numerous interesting features, the most distinguished of them are; its pure cellulosic form, high crystallinity, high water content and its high mechanical strength. All BC features are summarized in Table 2. The more studies and investigations are made, the more biocellulose is used in different kind of applications: not only in food industry but also in health care, cosmetics and beauty as well as in audio products [41]. One of the most commercial successes has achieved *Nata de coco* in health food industry. This desert is well known not only in Philippines, where it was originally made, but worldwide (especially in United States). Over the years strains able to utilize sucrose efficiently were selected to enable low cost utilization of abundant substrates. The one of the advantages of *Nata* culture is that a cellulose producing strain can be kept for a very long time.

Table 2. Summary of biocellulose features [32]

Purity:	The only synthesized biopolymer
	No presence of hemicellulose or lignin
	Biodegradable
High mechanical strength:	High crystallinity
	High tensile strength
	Light weight
	Noticeable durability
Remarkable absorbency in the hydrated state:	Extraordinary capacity to hold water
	Selective porosity
	High wet strength

In the past few years a significant increase of interest in biocellulose in green biomaterials applications is observed. BC is one of the most promising classes of biopolymer and its potential is lately used in biomaterial filed and in medical applications [42]. The most important feature is its biocompatible. It means that in contact with living tissues it does not cause any allergic or toxic side effects [43]. Furthermore, due to biocellulose importance in biomedical field, the number of investigations on mechanical properties increased [44]. The BC pellicles produced by *Acetobacter* are already used as a temporary tissues substitute for skin injury treatment. Because of its unique properties BC is shown to be highly effective wound dressing material [45]. This kind of products called Biofill for body burn, sutures, facial peelings, dermabrasion, skin lesions, chronic ulcers and skin graft. This kind of wound-healing system controls fluid loss and creates and maintains a moist environment in the wound, that is important in the healing process. The biggest advantage of biocellulose for those applications is its ability to be moulded into almost

every shape and size while synthesizing, without causing any significant change on its physical properties. It is important in treatment of difficult to cover parts of human body. In addition, BC is also suitable for use in micro nerve surgery and as an artificial blood vessel suitable for microsurgery with inner diameter of about 1 mm [46]. This product known as a BASYC-BActerial Syntffhesized Cellulose, was form as a result of cooperation among chemists, biologists and surgeons. The compatibility with smooth muscle cells was checked and obtained results revealed possibility of BC usage in tissue engineering blood vessels (TEBV) [47].

2.2 Effect of Feedstock on BC Biosynthesis

A carbon and nitrogen sources are considered to be essential components in the feedstock to produce BC. The cost of BC nanofibers production can be reduced by using the proper feedstock. The Hestrin S. (HS) medium used for BC cultivation was found to be too expensive and unsuitable for the commercial production of BC [37]. Corn steep liquor (CSL) and fructose medium was used by Toyosaki et al. and a high yield of BC was obtained compared to the conventional medium of yeast extract/peptone and glucose [48]. Several other studies found that the biosynthesis pathway of *G. Xylinum* produce higher amount of cellulose in the presence of corn steep liquor [49-51]. Bae & Shoda reported high BC production at a low cost by using molasses (MO) and CSL as the carbon and nitrogen sources [16]. Molasses is the most popular carbon source used in the fermentation industry in general. Keshk & Sameshima, reported that MO is a better carbon source than glucose for BC production in Hestrin medium using different strains of *Gluconacetobacter Xylinum*, and no significant difference in BC crystallinity among the results [52].

Hong & Qiu investigated konjac powder hydrolysate an alternative carbon source for the fermentation. Konjac and konjac powder are commonly available agricultural products in China. They found that hydrolysate of konjac powder is an excellent carbon source for BC production. The use of hydrolysates produced three times higher BC production than that when mannose sugar was used and five times higher than that when used a glucose-mannose carbon source mixture. Son et al showed that adding several inorganic salts to the culture medium increase the production of BC nanofibers. The cultivation medium contained glucose, ethanol and inorganic salts in distilled water. Eight days of cultivation produced 4.16 g/L of BC while agitated at 200 rpm [53]. They also tested the effect of Nicotinamide concentration on BC production, which they found that 0.00005% Nicotinamide (by volume) in the medium produced the highest amount of BC nanofibers. The addition of calcium cations is also proven to improve the BC production in static cultures.

Plants with high levels of xanthine-based substances (like caffeine) can be used as stimulators for BC production by *G. Xylinum*. These plants can be used as supplements in culture medium, they are commonly cheap and small quantities are enough for BC production. The examples are *Theobroma* (cacao), Ilex ("mate"), Paulinia (Guarana), *Camellia* (commercial tea), kola or sterculia (cola nut), and coffee (unroasted seeds). The best BC yield was obtained with Paulinia and tea infusions (*Camellia sinesis*), 295.5 mg % dry weight (per 100 mL of liquid and non agitated medium for short cultivation times of less than 5 days) for Paulinia, and 310.4 mg % dry weight (per 100 mL of liquid and non-agitated medium for long cultivation time ~ 15 days) for tea [54].

Vandamme et al. [55] produced BC in a static culture and in an agitated submerged fermentation process using micro particles with precise control of pH. Glucose and sucrose are

used as carbon sources, and found higher yields of BC production. They found that BC formation could be increased by adding insoluble micro particles, such as diatomaceous earth, silica, small glass beads, to the submerged, agitated/aerated *Gluconoacetobacter* culture. The combined use of fructose (70 g/L), glucose (35 g/L), and acetic acid (7.5 g/L) in a static culture produced 28.4 g/L of cellulose. The cellulose yield in the media to which micro particles were added was more than tripled compared to the level normally obtained in the similar reference cultures (without micro particles). This micro carrier-enhanced cellulose synthesis was believed to be the result of developing an oxygen-limiting biofilm around these particles [55]. Kouda at al considered the inhibitory effects of the partial pressure of carbon dioxide (pCO_2) on BC production. The study was done using *Gluconoacetobacter Xylinum*. The study included a 50-L jar fermenter that was purged with air containing pCO_2 (0.15-0.20 atm) and found that pCO_2 decreased the BC yield, volumetric production rate, and viable cells concentration [56].

Zhou et al studied the effects of sodium alginate in shake flasks and in a stirred-tank reactor. The strain used was *Xylinum Nust* 4.1. They found the maximum BC production of 6.0 g/L occurred in the shake flask at 0.04% (w/v) of sodium alginate and the BC production without sodium alginate was 3.7 g/L. However, the trend in improving the BC production by adding more sodium alginate was not observed. This trend suggests that higher concentrations of sodium alginate may impede BC production because of an increase of broth viscosity. Without sodium alginate, the BC was produced as clumps of different sizes, fibrous shapes, and was entangled with the internals of the reactor. However, with 0.04% (w/v) sodium alginate, these problems did not occur. The maximum yield of BC reached in the STR was 1.89 g/L for 60 h at a sodium alginate concentration of 0.04%. This yield was 1.7 times greater than the 1.09 g/L produced in the control medium without sodium. The BC production by *G. Xylinum Nust* 4.1 in the stirred-

tank reactor was much lower than that in shake flasks, because of the higher viscosity of the culture broth in the reactor [57,58].

2.3 Agricultural Biomass as Substrate

Common types of agricultural residues and wastes used for the production of biomaterials include wood (hardwood), wheat straw, corn fibers, rice straw, dried distillers‘ grains with soluble (DDGS), pulp and paper wastes [59-61]. As shown in Figure 4, agriculture biomass is composed mainly of three biomaterials; cellulose (35-48 % of dry weight), hemicellulose (22-48%) and lignin (15-27%) [5,62]. Their matrix composite is called lignocelluloses, a composite material of cellulose fibers embedded in a cross-linked network of hemicellulose and lignin that bind the fibers.

As mentioned before cellulose is composed of a highly crystallized linear polymer composed of thousands of glucose polysaccharides linked together in a tightly packed structure which make it very hard to decompose. Hemicellulose is considered easy to hydrolyze because of its amorphous polymer composed of xylose mainly beside minor sugar content of arabinose, galactose, mannose and others. Finally, the composition of lignin is non-carbohydrate based and composed of different non fermentable alcohols such as coniferyl alcohol, p-coumaryl alcohol, and sinapyl alcohol [32,63,63]. While the decomposition of cellulose and hemicellulose increase the fermentable sugars for cultivation, the liberated in the degradation of lignin are considered microbial inhibitors of fermentation [65]

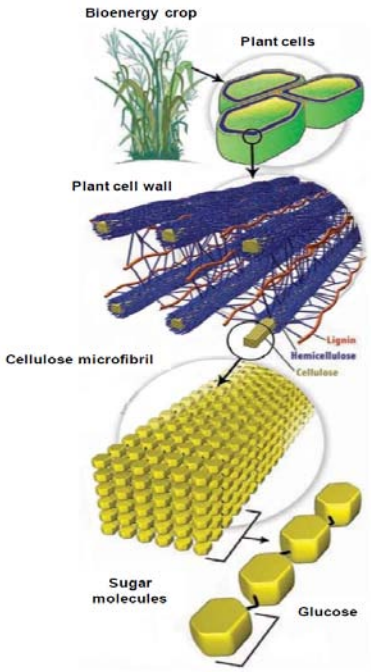

Figure 4. Agriculture crop composed of three major constituents: cellulose, hemicellulose and lignin [66]

Producing commercial products through fermentation of lignocelluloses is a multi-step process: It starts with pretreatment of the lignocelluloses to release fermentable simple sugars, then the fermentation of simple sugars by living microorganisms to produce hydrocarbons such as biopolymers or alcohols, and lastly recovery from the fermentation broth of the desired fermentation products, and utilization of the by-products. Although the process steps are described separately, the steps may be integrated to improve production performance [67]

2.4 Pretreatment and Hydrolysis

The purpose of pretreatment is to break the lignin and hemicellulose, reduce cellulose crystallinity, and increase the porosity of the materials [20]. The pretreatment often requires a combination of physical, chemical, and heat treatments to disorder the structure of cellulose and

hemicellulose then convert it into a more hydrolysable form. The complete depolymerisation of this renewable feedstock is a cost-effective process with minimal formation of degradation products. Various pretreatment methods such as the use of dilute acid, hot water controlled pH, and ammonia are now available to solubilise and depolymerise biomass [68,69]. Dilute acid pretreatment methods generate significant microbial inhibitors, while hot water and alkaline pretreatment methods generate only low concentrations of inhibitors [63,70]. Also, enzymatic hydrolysis is another favourable hydrolysis techniques being used without generating high inhibitors concentrations, since these enzymatic hydrolysis is usually conducted at mild conditions (pH 5.0 and temperature 30 to 45°C). During the enzymatic hydrolysis, the hemicellulose and cellulose components of the biomass are degraded to reducing sugars that can be fermented by bacteria to desired products [71].

3. MATERIALS AND METHODS

3.1 Materials and Chemicals

The following chemicals were ordered 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, Hydroxymethyl Furfural (5-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 ($NaMoO_4.2H_20$), Zinc Slfate Heptahydrate ($ZnSO_4.7H_2O$), Thiamine Hydrochloride, and *D-(+)*-Xylose.Appendix A shows the full list of supplier catalog numbers of the chemicals used.

Wheat Straw (WS) was collected from a local farm in Barrie, Ontario, and Corn steep liquor (CSL) was provided by Casco, London, Ontario, upon request, and used as received. Finally *Gluconoacetobacter Xylinum* (ATCC 700178) was supplied by American Type Culture Collection (ATCC), Manassas, VA 20108, USA.

3.2 Experimental Procedures and Methodology

3.2.1 Pretreatment of Wheat Straw

Wheat straws were initially subjected to physical pretreatment in which they were grounded to fine particles using a hammer mill (Retsch GmbH Inc. USA, model # 12930143D), and filtered by 1.0 mm pore size sieve screen. WS were then subjected to different conditions of chemical and thermal pretreatment, followed by enzymatic treatment.

Pretreatment of all samples in the present work was applied on 20 g of WS suspended in 250 ml liquid volume. Pretreatment conditions included chemical pretreatment (dilute sulphuric acid at different ratios (0-2% v/v), and soaking times (0 and 3 hours)); thermal treatment with different temperatures (121 and 135^{o}C) and heating times (30 and 90 minutes). Table 3 summarizes the different pretreatment conditions of samples in SHF method. The effects of heating time and temperature were examined in different fermentation media by samples F1, F6 and F7 (pretreatment in 1% dilute acidic solution), and samples F8, F9, and (pretreatment in water solution). Moreover, the effect of higher acidity concentration and soaking time were examined by samples F1, F2, and F3. Sample F3 was left at room temperature for 3 hours after pretreatment before filtering the WS solid materials from the solution. This method was called "soaking" process. At the end of pretreatment stage, the hydrolysate solutions in SHF method were finally recovered from the solid WS content by centrifugation at 4000 rpm for 30 min (accuSpin^{TM} 400, Fisher Scientific) and were further purified by a vacuum filtration using 0.45 μm pore size Pyrex glass.

Table 4 summarize the different pretreatment conditions of samples in SSF method. The effects of heating time and temperature were examined in different fermentation media by samples S1, S5 and S6. Moreover, the effect of higher acidity concentration was examined by samples S1, and S2. Following the chemical pretreatment, samples F4 and F5 in SHF method, and samples S3 and S4 in SSF method were subjected to enzymatic treatment. Enzymes *Cellulase* and *Beta-glucosidase* were used in the four samples, while enzyme *Xylanase* was further used in samples F4 and S3. An amount of 0.375mL of each enzyme was used with each sample, which then was stored at 45°C for three days. In SSF method, a syntactic polyester fine mesh was used to store the WS in it and keep it from mixing the produced BC. The choice on

polyester was because of its degeneration conditions are far from the operation conditions used in this study [72-74]. This option in SSF allowed continuous presence of WS in the solutions throughout the fermentation period.

Table 3. SHF method conditions of pretreatment of the different WS samples prepared prior to fermentation experiments

Sample	Chemical Treatment		Thermal Treatment		Notice
	Acidic (v/v) [a]	Soaking Time (hr) [b]	Temperature (°C)	Time (min)	
F1	1	0	121	30	Control
F2	2	0	121	30	High acid concentration
F3	1	3	121	30	Soaking for 3 hours
F4	1	0	121	30	Enzymatic treatment [c]
F5	1	0	121	30	Enzymatic treatment [d]
F6	1	0	121	90	Longer thermal treatment time
F7	1	0	135	30	Higher thermal treatment temperature
F8	0	0	121	30	Water pretreatment, reference
F9	0	0	121	90	Water pretreatment, Longer time
F10	0	0	135	30	Water pretreatment, Higher temperature

[a] *Total volume of 250 mL with 1% or 2% H_2SO_4*
[b] *WS soaked in acidic solution at room temperature before thermal treatment*
[c] *At 45°C for three days with0.375 mL of Cellulase, Beta-glucosidase and Xylanase enzymes*
[d] *Treated at 45°C for three days with 0.375 mL of Cellulase and Beta-glucosidase enzymes*

Table 4. SSF method conditions of pretreatment of the different WS samples prepared prior to fermentation experiments

Sample	Chemical Treatment Acidic (v/v) [a]	Thermal Treatment		Notice
		Temperature (°C)	Time (min)	
S1	1	121	30	Control
S2	2	121	30	High acid concentration
S3	1	121	30	Enzymatic treatment [b]
S4	1	121	30	Enzymatic treatment [c]
S5	1	121	90	Longer thermal treatment time
S6	1	135	30	Higher thermal treatment temperature

[a] *Total volume of 250 mL with 1% or 2% H_2SO_4*
[b] *At 45°C for three days with 0.375 mL of Cellulase, Beta-glucosidase and Xylanase enzymes*
[c] *Treated at 45°C for three days with 0.375 mL of Cellulase and Beta-glucosidase enzymes*

3.2.2 Bacterial Strain and Culture Growth Conditions

G. Xylinus bacterial strain (ATCC 700178) was used as the BC producing bacteria in all the samples of this study. This strain is widely used in literature as the best yield BC producing bacterium [75,76]. The metabolism of *G. Xylinus* can utilize different sugars as a carbon source, where all the sugars available in WS composition can fully or partially utilized in BC production [64,76]. *G. Xylinus* bacteria 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, Figure 5.

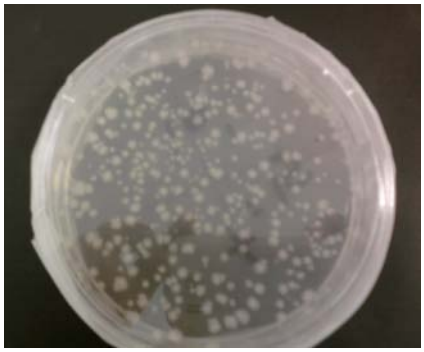

Figure 5. Bacterial colonies after planting on a Petri dish for 7 days

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.

3.2.2.1 Working under aseptic conditions

Aseptic culture handling were done in a laminar flow biological safety hood (Labgard, class II, type A2, Nuaire). Every time the bench was used, the air flow blower was turned on 5-10 min before starting any work. Then the entire surface was cleaned with 70% ethanol spray and tissue, and then followed by 10-15 minute UV lamb disinfection. All the used tools such as Petri plates, parafilms, burner, inoculation loop, spatula, cell spreader, needles, pipettes and tips were kept under UV for disinfection. Before work, hands were washed thoroughly with anti bacterial soap and warm water, and then disposable medical gloves were used. All metallic tools

used inside the hood (needles, loop, spatula and cell spreader) were flamed until red-heated and cooled before use. While working under aseptic conditions, the protective glass of the hood was left open to the minimum, just to allow enough working space.

3.2.3 Production of the Biocellulose Nanofibers

Biocellulose productions were conducted in 500 mL shaking flasks each containing 200 mL of fermentations medium. The media composition was as follows: 195 mL of WS hydrolysate solution in SHF or suspension of 20g in 195 mL of water in SSF (Carbon source), 5 mL of CSL (nitrogen source), 1 g/L of KH_2PO_4, 0.25 g/L of $MgSO_4$.7H2O, 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 Lhiamine Hydrochloride [10,77].

All glassware was sterilized in an autoclave (Sanyo MLS 3780) at 121°C for 10 min prior to use. Hydrolysate solutions and their additives were sterilized separately from CSL, each at 121°C for 10 min with initial pH 5.0 to prevent high temperature reaction of sugars and amino acids (Maillard reaction) at which produce black nitrogen containing compounds that impede microorganisms' growth [78]. CSL was aseptically added to the hydrolysate additives mixture and sterile distilled water was added to compensate for evaporated water during autoclave if necessary. When the solutions reached room temperature each flask was aseptically inoculated using 2 mL of the inoculum, and the tip of the flask covered with a sponge that allow oxygen transfer, 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.

3.2.4 Sampling

During enzymatic treatment (samples F4, F5, S3 and S4) periodic 2 mL samples of hydrolysate were collected using 0.45 μm syringe filters (Gelman Acrodisc CR PTEF, Millipore). In addition 2 mL of hydrolysate solutions were collected after filtration at the end of each WS pretreatment. In all fermentation experiments, samples of 2 mL were first taken after inoculation and periodically thereafter till the end of fermentation. These samples were collected under aseptic conditions in the biosafety hood. When collecting samples from fermentation flasks, the tip of the flask was kept above the flame right after opening the bottle sponge till the sample was taken. This was done for extra safety in order to kill any bacteria living on the surface of the flask tip. At the end all the fermentation flasks were brought back into the incubator to continue the fermentation process. All the collected samples were stored at -80°C until analyzed.

3.3 Analytical Techniques

3.3.1 pH measurement

For each of the prepared solutions the pH of the medium was set to 5.0 before bacterial inoculation, and measured at the end of the 7 days fermentation period. Before any pH measurement the pH meter (Easy Seven, Mettler Toledo) was calibrated to get the most accurate results. The calibration was made according to manual provided by the producer. Three standard buffers were used: pH equal to 4.0, 7.0 and 10.0.

3.3.2 Measurement of sugar concentration

Sugars concentrations were measured using high performance liquid chromatography (HPLC-Perkin Elmer) equipped with a refractive index detector (2414, Waters) and automatic sample injector (Figure 6). Two HPLC columns were used separately, Shodex SP0810 for measuring sugars concentration and Shodex KC811 for measuring inhibitors concentration. Mobile phase solvent was 5 mM H_2SO_4. The water used in preparing the solvent was autoclaved at 121°C for 15 min, and then filtered using 0.2 μm PTFE- filters (Whatman, USA) and double distilled. The solvent was then degassed using an inert gas followed by using vacuum filtration. All the samples were centrifuged at 15000 rpm for 15 min and double filtered through 0.2 μm PTFE- filters.

A blank sample with only double distilled water was applied in the first sample vial. This blank was used to increase the flow rate of the solvent from 0 to 0.6 ml/min. The flow rate was maintained at 0.6 ml/min for 1 hour while increasing the temperature of the HPLC column from 20°C to 60°C. This also fixes the pressure at a constant value and cancels most of the noise generated during the analysis. Then, each sample vials were arranged in a sequence and 0.1 μl of sample was extracted by the automatic sample injector. Each sample was analysed through the HPLC for 30 min. Data was processed by the computer software (Turbochrom Navigator). It was important to fill the HPLC testing vials to a minimum headspace to reduce the loss of solvents in vapour phase. Concentrations were obtained from the area under correlated peak using previously constructed calibration curves (Appendix B). The reliability of HPLC column, and testing parameters were confirmed by running standard samples in triplicates

Figure 6. HPLC (model # 600 by Perklin Elmer) equipped with refractive index (model # HP1047 A, Hewlett Packward)

3.3.3 Cell count measurement

3.3.3.1 Dry cell weight

The dry weigh of bacterial cells was used in yield calculations in Tables 6 and 8. Sample from each fermentation medium was collected right after cells inoculation. The sample was centrifuged to precipitate all the cells in the liquid and repeatedly washed two times with distilled water. The suspended cells were then transferred into a pre weighted crucible and dried at 80°C till it reached a permanent fixed weight. The crucible was then weighted and back calculated to cells dry weigh in the fermentation solution.

3.3.3.2 Cells concentration count

Viable cell counting was done by using a hemocytometer (QiuJing XB-K-25) occupied with two counting chambers each having 1/400 mm^2 unit area and 0.1 mm high. Counting was done under optical microscope (Zeiss Axio Observer A1) at 50X magnification. The haemocytometer was autoclaved at 121°C and kept under UV for 10-15 minutes for disinfection before counting. The chambers were cleaned with 75% ethanol, rinsed with distilled water and dried after each sample measurement. Different dilution factors were used depending on the cells

concentration of the samples. Three separate quadrant volumes were counted for each sample, and the final average was used to calculated cells concentration. When the error of three counts was sensibly high more counts were performed until the error is less than 5%.

Equations 3.1 and 3.2 were used in calculating the cells concentrations

$$\frac{\text{Average cells}}{\text{Chamber}} = \frac{Quadrant\ 1 + \ Quadrant\ 2 + \ Quadrant\ 3\ (cells)}{3} \times \frac{4\ \text{Quadrants}}{\text{Chamber}} \qquad (3.1)$$

$$\text{cells concentrations}\ \left(\frac{\text{cells}}{\text{mL}}\right) = \frac{\text{Average cells}}{\text{Chamber}} \times \text{dilution} \times \frac{\text{Chamber}}{\frac{1}{400} \times 0.1\ \text{mm}^3} \times \frac{1\ \text{mm}^3}{0.001\ \text{mL}} \qquad (3.2)$$

To check the reliability of this method, the cell count of random samples was dose by serial dilution method as a more traditional approach. Samples Dilution in the range of 10^5 were prepared and 0.1 ml was plated onto agar plates and left at its appropriate conditions over night to form colonies. Each colony represents one cell in the diluted sample. The number of colonies were counted and multiplied by the dilution rate and expressed in cells/ml.

3.3.4 Measurement of BC production

After 7 days of incubation, the reaction medium was treated with excess 2 N NaOH at 100°C for 5 min to kill the bacterial cells. Then, 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 25 mL by adding distilled water, and the solution homogenised in a grinder (Kenmore) for 15 sec. Subsequently, 1 mL of the sample was transferred to a previously weighted crucible and placed in an oven at 80°C for one day to dry to a permanent constant weight, the crucible containing

dried sample was then returned to room temperature and weighted. All results presented are the average of triplicate, with average error of less than 5% for each sample.

3.3.5 Reproducibility of experimental data and error analysis

Each experimental procedure was run in triplicates. Also, each date point presented in the results and discussion section is an average of the measurement of these repetitive triplicates, with the relative standard deviation listed on each table or figure of data. Appendices B and C list all the raw date of SHF and SSF methods respectively for which the calculation procedure is illustrated in the analytical techniques section.

Equations 3.3 and 3.43 were used to calculate standard deviation (STDEV) and percent relative standard deviation (%RSD) respectively.

$$STDEV = \sqrt{\frac{\sum(X - \bar{X})^2}{(n-1)}} \tag{3.3}$$

$$\%RSD = \frac{STDEV \times 100}{\bar{X}} \tag{3.4}$$

Where, $\bar{x}$ = sample mean value; x = data point n = sample size

The average error in every set of data presented was less than 5% in all collected experimental data.

4. RESULTS AND DISCUSSION

4.1 Production of BC in SHF

4.1.1 Final BC production and pH

Figure 7 presents the total BC production using SHF method, and the corresponding pH of the media at the end of fermentation period for the different samples tested. The conditions used in each sample are summarized in Table 3.

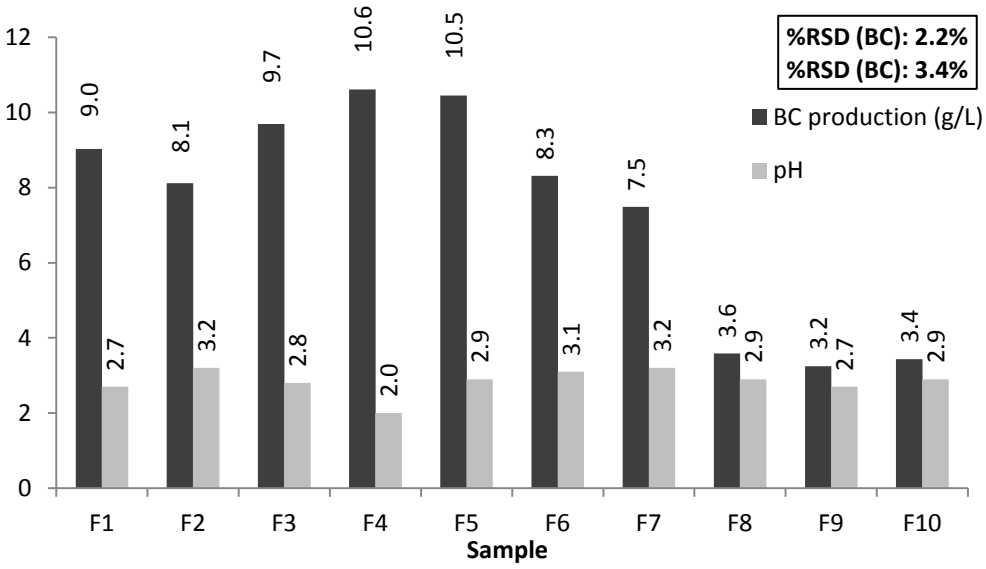

Figure 7. Total BC production and final solution PH following the SHF production method.

Total BC production in Table 7 was the range of 7.5 and 10.6 g/L for acidic pretreated samples (F1 through F7), with pH of the media at the end of fermentation in the range of 2.0 and 3.2. Further examination of the results in Table 7 shows that the maximum BC productions were obtained in samples F4 and F5 generated 10.6 and 10.5 g/L of BC, respectively. Enzymes were used in these two samples during the WS pretreatment stage. Samples F1 and F3 produced total BC of 9.0 and 9.7 g/L respectively. Both samples were pretreated under the same conditions,

except that WS in sample F3 was soaked for 3 hours during pretreatment, while sample F1 was treated with dilute acid without any further soaking. Samples F2, F6 and F7 achieved BC production in the range of 7.5 and 8.3 g/L, which represents the lowest BC production among all acid pretreated samples in Table 7. These samples were pretreated with high acidic solution in sample F2, and higher temperature thermal treatment in samples F6 and F7. Samples F8, F9, and F10 that were all subjected to water pretreatment, produced lower total BC amounts of ~3.4 g/L. Apparently, pretreatment at higher temperature and longer times that were applied in the pretreatment of samples F6, F7 and F9 had limited effect on the final BC production when comparing samples F6 and F7 to sample F2, or F9 and samples F10 to F8. Interestingly, the final pH of solutions measured at the end of fermentation showed that all samples reached pH levels between 2.7 and 3.2, except sample F4 that had lower pH of 2.0 at the end of fermentation. According to Yang et al. [79] and Bedran et al. [80], *G. Xylinus* bacterium metabolized part of the polysaccharides to produce gluconic acid or acetic acid that increases the acidity of the fermentation broths and leads to reducing the final pH.

4.1.2 Change in sugars concentrations

Concentrations of individual sugars produced during WS pretreatment and the sugars left unconsumed at the end of fermentation are presented in Table 5. Results shows that produced sugars under the different pretreatment conditions were mainly composed of the glucose and xylose. Average percentages of glucose and xylose among the other individual sugars in Table 5 are 56 and 28%, respectively. Moreover, individual sugars of galactose, manose and arbinose formed ~15% of the total sugars. The high glucose and xylose concentrations in all samples come from WS dry basis (39% cellulose and 32% hemicelluloses), which are primarily composed of glucose and xylose [5,62,81]. Nevertheless, the higher xylose and glucose

concentration in samples F4 and F5 occurred due to the utilization of the enzymes *Xylanase*, *Cellulase*and *Beta-glucosidase* in the pretreatment of WS, which allowed for further hydrolysis of the WS once compared with dilute acidic samples. Although fructose exist in the dry basis of WS [64], and is known to promote BC production [13,82], no indication of its presence in the hydrolysate was obtained compared to all other individual sugars in Tables 5 (i.e., less than 0.1%). This was clearly marked in several previous WS hydrolysis investigations that demonstrated the absence of fructose in the final hydrolysate sugar composition [83,84].

Examining results in Table 5 reveals that 25% of glucose was unconsumed and remained at the end of fermentation in all samples. The corresponding percentage that was observed for galactose, manose and arbinose was ~ 28%. The lowest consumption rate was obtained with xylose, with 35% of its initial amount was unconsumed towards the end of the fermentation. Generally, the superior BC production that was obtained with samples F1 to F7 can be explained by the higher glucose contents (i.e., 20 to 28 g/L compared to ~13.5 g/L in the remaining samples). The high consumption of glucose in all samples occurs as it is the basic component of the produced BC polymer and also consumed in energy production metabolism of *G. Xylinus* [77]. Meanwhile, the closely equal consumption of galactose, manose and arbinose is due to their initially low concentrations in Table 5, given their repeatedly reported high BC yield by stabilizing the pH of fermentation [13]. The lower percentage of xylose consumption indicates the complicity of its metabolism, while the majority of this sugar is consumed to boost cells' growth and not for BC production [82,85]. The effect of pH diverging from the optimum 4-5 level implies that it might have prevented BC production from reaching higher concentrations. The increase in solution acidity derives *G. Xylinus* metabolism to undesired side products [79, 80]. This demonstrates potential to improve production of BC when employing pH control at larger scale.

Table 5. Concentration of individual sugars there were produced from hydrolysis pretreatment at the beginning and the end of fermentation (%RSD: 2.2%)

Samples	Individual sugars present at the beginning of SHF (g/L)					Individual sugars left at the end of 7 days SHF (g/L)				
	Glucose	Galactose	Mannose	Xylose	Arbinose	Glucose	Galactose	Mannose	Xylose	Arbinose
F1	19.43	2.02	1.53	9.79	3.41	2.63	0.50	0.41	3.02	0.94
F2	22.74	2.21	2.00	11.89	3.76	5.88	0.86	0.69	4.56	1.20
F3	22.89	2.25	2.02	12.33	3.53	4.98	0.56	0.54	3.78	0.96
F4	27.85	2.99	2.25	15.06	3.97	5.40	0.70	0.57	4.46	1.02
F5	28.75	2.04	1.53	9.79	3.42	5.17	0.40	0.34	2.74	0.86
F6	21.58	2.07	1.84	10.69	3.74	7.84	0.74	0.59	4.20	1.21
F7	20.58	2.09	1.78	10.46	3.45	7.90	0.80	0.62	4.25	1.17
F8	13.45	0.04	0.04	6.52	1.78	2.63	0.01	0.01	1.76	0.44
F9	13.5	0.03	0.03	7.54	2.38	3.60	0.01	0.01	2.28	0.68
F10	14.04	0.04	0.04	7.27	2.06	3.46	0.01	0.01	2.25	0.63

The change in the percentage of total sugars concentration with fermentation time is illustrated in Figure 8. Figure 8 shows high total sugars consumption in all solutions during the first 35 hours, as they declined exponentially till the end of fermentation. After 50 hours of fermentation ~35% of the sugars were consumed in samples F2, F6 and F7, whereas 55% to 65% of total sugars were consumed in the same time in the rest of the samples. Sugar consumption

rate observed to decrease significantly with time. After 100 hours of fermentation less than 0.1% change per hour was observed in all samples. At the end of fermentation samples F2, F6 and F7 had around 40% unconsumed sugars, whereas the rest of the samples had between 20 and 30% of unconsumed sugars.

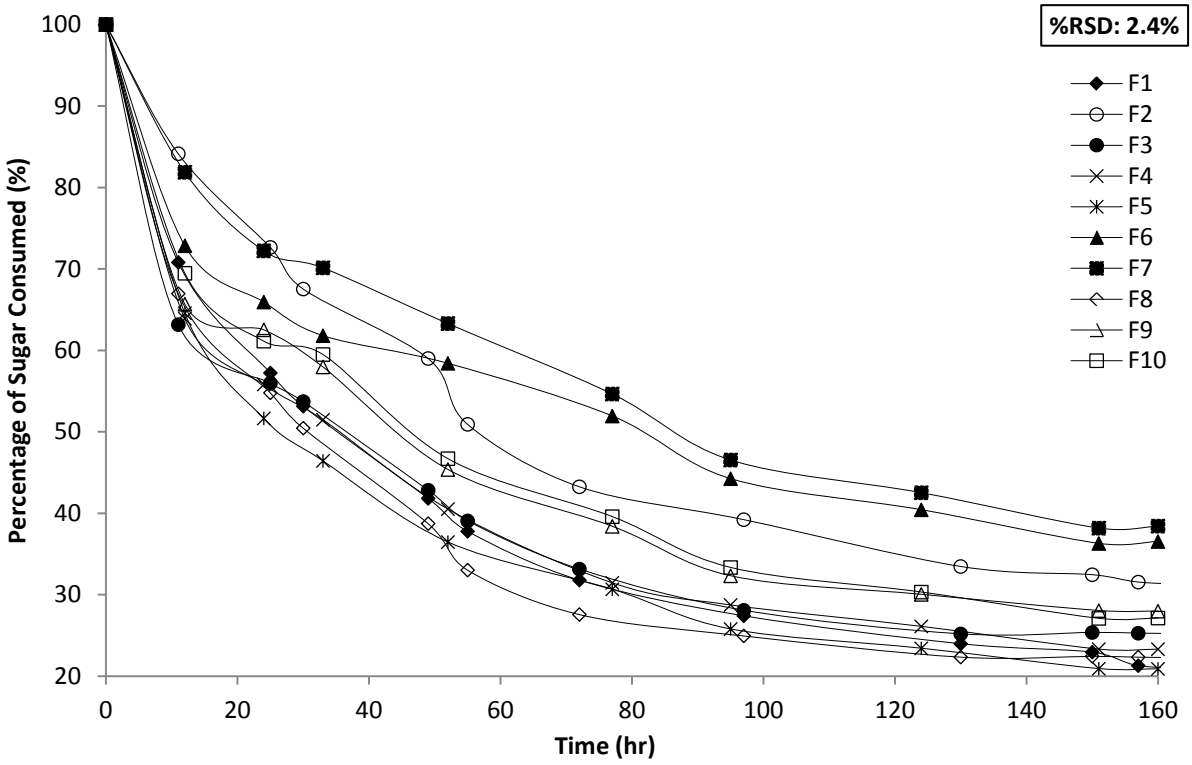

Figure 8. Changes in the percentage of total sugars concentrations in the fermentation medium during SHF for all samples

4.1.3 Change in fermentation parameters

Table 6 shows the main variable parameter in the SHF reaction. The first two columns illustrate the difference between the total sugars produced during the pretreatment of WS, and the total sugar consumed during fermentation for the different samples.

Table 6. Fermentation kinetic parameters of *G. Xylinus* during SHF for all samples

Samples	BC Production (g/L)	Total Sugars (g/L)		Average cell concentration (10^7 cells/mL)	Average cell proliferation rate (10^4 cells/mL.h)	Cellulose Yield		Furfural (g/L)
		Available	Consumed			$Y_{P/C}$ [a]	$Y_{P/S}$ [b]	
F1	9.0	36.19	28.69	5.26	55.53	58.61	0.31	0.30
F2	8.1	42.59	29.4	3.90	33.58	64.22	0.28	1.24
F3	9.7	43.02	32.2	6.10	52.86	64.58	0.3	0.30
F4	10.6	52.12	39.97	6.85	83.14	67.17	0.27	0.31
F5	10.5	45.53	36.02	6.40	70.94	57.53	0.29	0.30
F6	8.3	39.92	25.33	2.92	31.44	55.36	0.33	1.57
F7	7.5	38.36	23.62	2.60	27.48	48.59	0.32	1.81
F8	3.6	21.84	16.99	1.63	12.51	23.87	0.21	0.20
F9	3.2	23.49	16.92	1.13	9.63	21.63	0.19	0.23
F10	3.4	23.46	17.09	1.19	11.07	22.86	0.2	0.30

[a] *Weight of BC produced to dry cell weight at the beginning of fermentation (g product/ g cells).*
[b] *Weight of BC produced to weight of total sugars consumed (g product/ g sugars).*

Results in Table 6 show that sample F1 produced 36 g/L of total sugars during the pretreatment step, whereas water pretreated sample F8 that was subjected to similar thermal treatment of 30 minutes heating at 121°C produced 22 g/L total sugars. The difference is also marked between samples F6 and F7 (both were pretreated with 1% dilute acid solution) and samples F9 and F10 (both were pretreated with water under similar thermal condition). On the

other hand, total sugars concentration in the hydrolysate increased by 6.5 g/L when the acidic solution concentration was increased from 1% in F1 to 2% in F2, or by soaking for 3 hours as in sample F3. Similarly, increasing heating time and temperature as occurred with samples F6 and F7 pretreatment resulted in less than 4 g/L increase in total sugars produced compared to that of F1 (all were treated with 1% acidic solution). On the contrary, water pretreated samples F9 and F10 were negligibly affected by increasing heating time and temperature compared to their standard reference of sample F8. The use of 1% dilute acid in the pretreatment of some of the samples increased total sugars extraction by more than 65% compared to that of water pretreatment. While less than 15% increase of total sugars was attained using 2% acidic solution instead of 1%.

4.1.4 Production of fermentation inhibitors

Two fermentation inhibitors were monitored in WS pretreatment since they have been reported to inhibit BC production in *G. Xylinus* metabolism, furfural and 5-hydroxymethyl-furfural (5 - HMF) [19,65]. Negligible amounts of 5-HMF were measured in all samples, whereas Table 6 shows furfural concentrations liberated during pretreatment. The negligible concentrations of 5-HMF measured are in consistence with previous studies on WS hydrolysis [83]. Meanwhile, all samples liberated 0.3 g/L or less of furfural, except samples F2, F6 and F7 which under extreme pretreatment condition liberated over 1.2 g/L of furfural. Samples F2, F6 and F7that are characterized by high furfural concentration (Table 6) exhibited slower sugar consumption during fermentation. Furfural is produced by dehydration of hemicelluloses when subjected to intensive heating in the presence of sulfuric acid [86]. This explains the increase of furfural concentration as acid concentration or boiling temperature and time were increased

during WS pretreatment as shown in Table 3. Table 6 apparently showed that furfural concentration higher than 1g/L considerably affected cells growth and proliferation. Similar furfural limits are also reported on its inhibitory effects in fermentation reactions [87]. The difference in consumed sugar in samples F2, F6 and F7 that was not used for cells growth or proliferation can be attributed to cells maintenance [11]. Furthermore, heating for longer time or at higher temperature in water samples F9 and F10 did not alter furfural concentration or inhibited BC production compared to sample F8. This signifies that furfural concentration does not reach inhibitory levels in the absence of dilute acids in the pretreatment solution [87].

4.1.5 Change of bacterium concentration

The results in Table 6 show dependency of cells growth rates and BC production on the quantity of sugar consumption. Samples F1 to F7 consumed between 23 to 40 g/L of sugars and produced between 7.5 and 10.6 g/L BC. On the other hand, samples F8, F9 and F10 consumed more than 70% of their initial sugar concentration (~17 g/L) and their BC production was less than 4 g/L because of the low sugar yield in water pretreatment (~22 g/L). Figure 9 illustrates the change in cells concentration during the fermentation process. As shown in Figure 9, the bacterial strain of *G. Xylinus* experienced a delay in growth between 30 to 35 hours for all samples except for F4, which increased rapidly after inoculation at time zero. After the delay phase, the cell concentration started to increase in all samples during the growth period. Samples F1, F3, F4 and F5 grew exponentially for more than 30 hours and reached between 6.5×10^7 and 12×10^7 cells/mL. While samples F2, F6 and F7 experienced a little shorter growth phase between 20 and 30 hours, but the cells concentration reached was between $3\times10^7$and 6×10^7 cells/mL. Next, the water-pretreated samples F8, F9 and F10 experienced a growth phase for less than 20 hours and reached final cell concentrations between 1.5×10^7 and 2×10^7 cells/mL (from Table 6).

Finally, after the growth phase the increase in cells concentration occurred in much slower rates and reached limiting values that slightly changed after the growth phase.

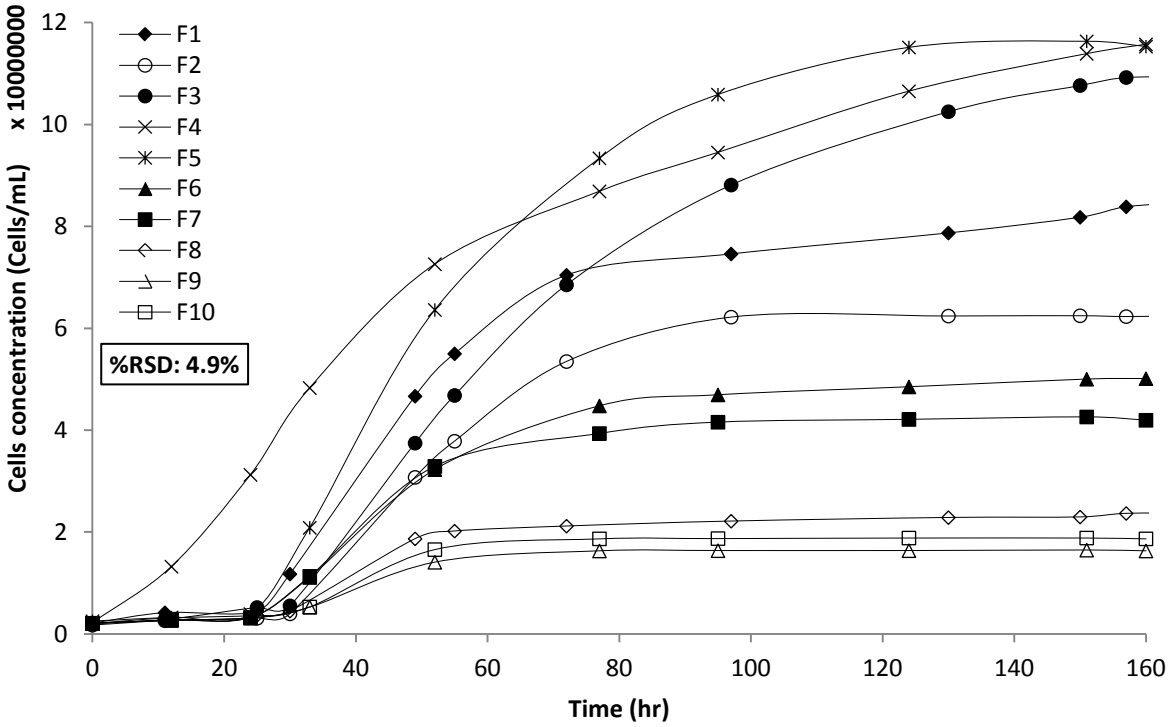

Figure 9. Change in *G. Xylinus* bacterial cell concentration during the SHF method.

The reason behind the quicker cells growth in sample F4 with relatively short delay phase is the high xylose concentration that accumulated due to the use of *Xylanase* enzyme during WS treatment. This again demonstrates the role of metabolized xylose in bacterial growth was relatively higher than production of cellulose [13]. This functionality of xylose also explains that the rapid drop in pH of F4 fermentation medium as it is metabolized for bacterial cells proliferation and oxidized to produce acetic acid that reduced the solution pH [79,82,86]. On the other hand, water- pretreated samples (i.e., F8, F9 and F10) did not attain high bacterial growth and ended with the lowest cell concentrations and low BC production. This could be due to the limitation of their water pretreatment method, which produced 50% less total sugars than acid

pretreated samples. Lastly, the effect of high furfural concentration in samples F2 and F6 and F7 on decreasing BC production in Figure 7, can be now reasoned to lower cells concentration in these samples than the rest of the samples which have comparable total sugars. This resulted in a shorter bacterial growth phase, and lowered the cells concentration during the stationary phase when most of the BC production took place [19,65,87].

4.2 BC Production in SSF

4.2.1 SSF Final BC production and pH

Figure 10 presents the total BC production using SSF method, and the corresponding pH of the media at the end of fermentation period. The conditions used in each sample are summarized in Table 4.

The highest BC production in Figure 10 was obtained in sample S1 with 10.8 g/L, this sample was pretreated with 1% dilute acidic solution at 121°C for 30 minutes. In addition samples S2, S5 and S6 achieved lower BC production in the range of 8.2 and 8.9 g/L. These samples were pretreated with high acidic solution in sample S2, and higher temperature thermal pretreatment in S5 and S6. Samples S3 and S4 produced total BC of 7.3 and 7.2 g/L, respectively, which represent the lowest BC production among all acid pretreated samples in Table 4. Enzymes were used in these two samples during WS pretreatment and throughout the SSF fermentation.

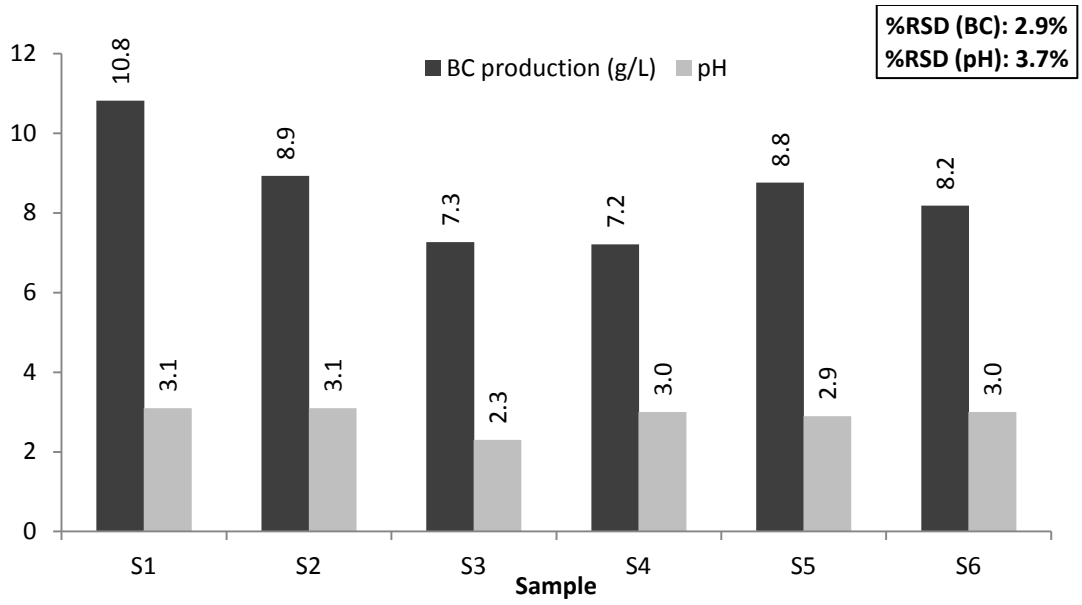

Figure 10. SSF method total cellulose produced and final pH for each sample

Apparently, samples pretreatment at higher acidic concentration (S2), longer time (S5) and higher temperature (S6), or the use of scarification enzymes (S3 and S4), had lower BC production than the optimum conditions applies in samples S1. This came in contrary with what was expected after studying the results of SHF method, as samples S3 and S4 treated with enzymes would have the highest sugars concentration and thus the highest BC feedstock in SSF method. The reason behind low production in enzymatic samples will be further discussed down in the analysis.

Interestingly, the final pH of solutions measured at the end of fermentation showed that all samples reached pH levels between 2.9 and 3.1, except sample S3 that had lower pH of 2.3 at the end of fermentation. This pH drop is due to the increase of gluconic acid or acetic acid in the medium [79]. SSF fermentation is reported to have better pH stabilization ability in the fermentation medium that result in higher production yield of the desired product [88]

4.2.2 Change in sugars concentrations

Concentrations of individual sugars produced during WS pretreatment (before fermentation) and that of unconsumed sugars at the end of fermentation are presented in Table 7. The results shows similar sugar composition in the pretreatment of SSF samples to those collected in SHF samples. The solutions before fermentation contained ~53% glucose, ~30% xylose, and the rest of individual sugars (galactose, manose and arbinose) formed ~16% of the total sugars. As in SHF method, minor quantities of fructose were measured in all the samples in the SHF method. The high glucose concentrations in samples S3 and S4 compared to the rest of the samples come from using enzymes *Cellulase* and *Beta-glucosidase* in the pretreatment of these samples. Moreover, the high concentration of xylose in S3 is due to using enzyme *Xylanase* in this sample. The similarities in the concentrations of individual sugars between SHF and SSF methods comes as the same WS pretreatment conditions were applied in the two fermentation methods. In addition, this prove that placing the WS inside a polyester mesh in the SSF method had negligible effect on the composition of sugars in the solution, and the polyester is self did not hydrolyze under the used conditions for WS pretreatment [72,74].

Table 7. Concentrations of individual sugars that were produced from WS hydrolysis before and after SSF (%RSD: 2.2%)

Samples	Individual sugars present at the beginning of SSF (g/L)					Individual sugars left at the end of 7 days SSF (g/L)				
	Glucose	Galactose	Mannose	Xylose	Arbinose	Glucose	Galactose	Mannose	Xylose	Arbinose
S1	19.49	2.04	1.53	9.78	3.42	1.73	0.26	0.21	1.55	0.48
S2	22.72	2.21	2.02	11.91	3.77	4.17	0.43	0.38	2.88	0.68
S3	27.78	3.02	2.25	15.07	3.99	6.81	0.93	0.77	5.72	1.24
S4	28.80	2.04	1.51	9.78	3.41	7.63	0.63	0.59	3.58	1.05
S5	21.61	2.08	1.85	10.66	3.76	4.86	0.46	0.36	2.52	0.73
S6	20.62	2.09	1.74	10.49	3.45	4.80	0.44	0.36	2.58	0.71

The concentration of individual sugars left at the end of fermentation shows that ~20% of glucose was unconsumed and remained at the end of fermentation in most of the samples. All the glucose concentrations at the end of fermentation were between 4.2 to 7.6 g/L, except sample S1 seems to consumed most of glucose in it with 1.73 g/L left in the medium after 7 days. Moreover, all the individual sugars in S1 had lower concentrations left at the end of fermentation compared to the rest of the samples. This high consumption of sugars can explain why S1 had the highest BC production among all sample in the SSF method, in correlation with the data in Figure 10. Meanwhile, samples S3 and S4 where pretreated under the same conditions as S1, and were further subjected to enzymatic treatment that extracted extra sugars from the WS. The drawback in BC production and decrees in individual sugars consumption in S3 and S4

compared to S1 suggest a limitation in the fermentation medium of S3 and S4 that prevents higher sugars utilization to produce BC.

The change in the total sugars percentage throughout SSF process is illustrated in Figure 11

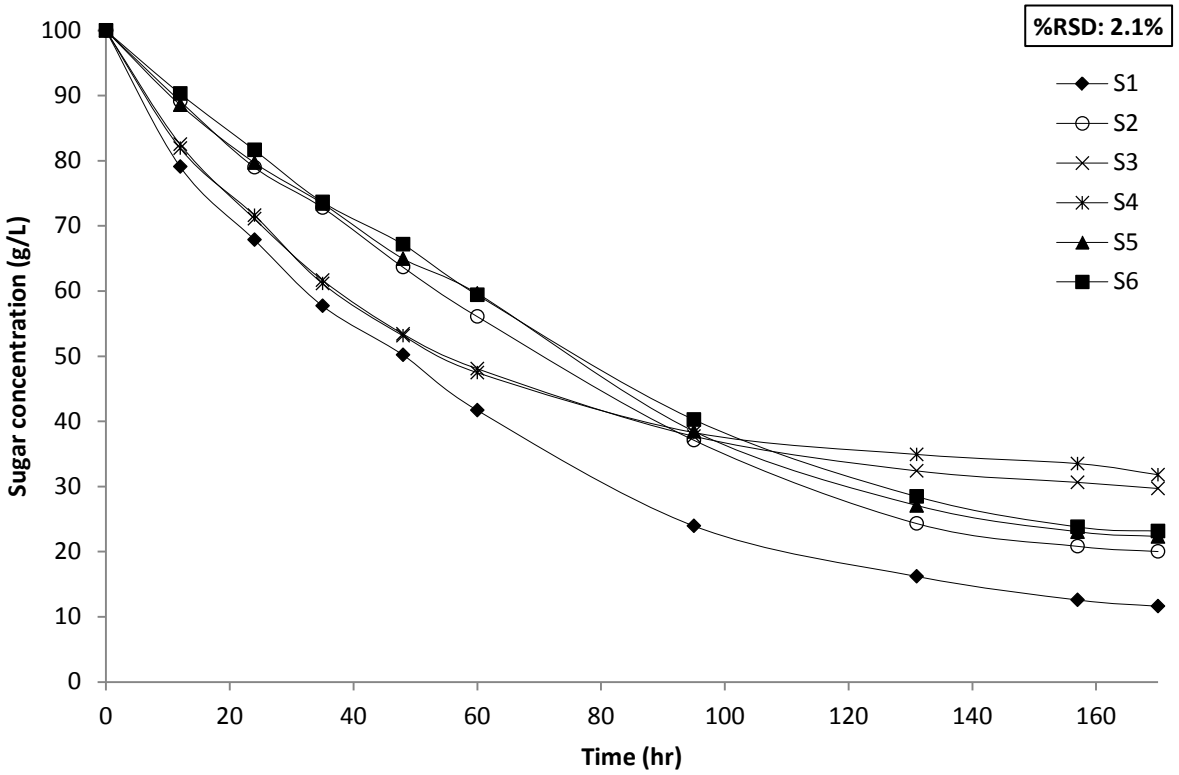

Figure 11. Changes in the percentage of total sugars concentrations in the fermentation medium during SSF for all samples.

The total sugars percentage change in Figure 11 shows two profiles of consumption during the first hours of fermentations. Samples S1, S3 and S4 had rapid sugars consumption in a short period of fermentation start up. Around ~50% of the sugars were consumed after 50 hours of fermentation in these samples. While, samples S2, S5 and S6 had slower sugars consumption, where ~35% of total sugars were consumed in the first 50 hours of fermentation. Samples S1, S3

and S4 were pretreated in 1% acidic solution at 121°C for 30 minutes, while the second group samples (S2, S5 and S6) were pretreated under extreme conditions such as; higher acid concentration (2% acidic) in S2, or longer boiling time (90 minutes) and temperature (135°C) in S5 and S6 respectively (Table 4). The slight difference in sugars percentage between S1 compared to S3 and S4 during the start-up would be since S3 and S4 were treated with enzymes after the pretreatment and hence had higher total sugars quantity.

While sugar percentages continued to decrease gradually with time in all samples, the pace of sugar consumption changed significantly toward the end of fermentation. Samples S3 and S4 had the least consumption after 90 hours of reaction, they reached the end of fermentation with ~30% sugars unconsumed. Then, samples S5 and S6 ended the 7 days fermentation with 20 - 25% unconsumed sugars. Sample S1 had the best sugar utilization in all stages of the reaction, and reached as low as ~10% of unconsumed sugars left unconsumed at the end of 7days.

The percentage of sugars consumed is directly related to the quantity of BC production in Figure 10. Sample S1 produced the highest BC as it consumed almost all the sugars available in the medium, whereas the rest of the samples had comparable BC production to their lower sugars utilization. It is expected that pretreatment conditions in samples S2, S6 and S7 produced inhibitory ingredients that limited higher utilization of sugars in the fermentation. Also, the rapid decrease in samples S3 and S4 sugars consumption (together with high glucose concentration in these samples) suggests that enzymes present in the medium might be hydrolyzing part of the produced biocellulose besides hydrolyzing the plant cellulose present in the WS.

4.2.3 Change in SSF parameters

Table 8 shows the main variable parameter in the SSF reaction.

Table 8. Fermentation kinetic parameters of *G. Xylinus* during SSF of all samples

Samples	BC Production (g/L)	Total Sugars (g/L)		Average cell concentration (10^7 cells/mL)	Average cell proliferation rate (10^4 cells/mL.h)	Cellulose 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	0.265	0.31
S2	8.93	52.87	44.33	3.94	50.34	57.95	0.233	1.21
S3	7.27	54.92	39.45	7.38	90.76	46.00	0.191	0.32
S4	7.21	54.04	40.56	6.40	88.99	39.67	0.197	0.31
S5	8.76	53.20	44.28	3.50	45.12	69.28	0.239	1.53
S6	8.18	53.52	44.63	3.27	42.00	54.48	0.216	1.82

[a] *Weight of BC produced to dry cell weight at the beginning of fermentation (g product/ g cells).*
[b] *Weight of BC produced to weight of total sugars consumed (g product/ g sugars).*

During SSF reaction part of the sugars extracted from WS is being directly utilized in the fermentation. This did not allow quantifying the total sugars available for fermentation in SSF method. Alternatively a set of blank of fermentation samples was conducted at the same conditions as SSF (WS quantity, solution acidity, enzymes, boiling time and temperature) but without the presence of fermentation bacteria which consumes the sugars. The total sugars accumulated in those samples were quantified after equal exposure time of WS as SSF method,

and the values reached were considered total sugars available for SSF samples fermentation (third column in Table 8).

Results in Table 8 shows that total sugars available for fermentation were closely equal in all SSF samples studied. Whereas, the values of consumed sugars varied among samples but still synchronized with the BC production in Figure 10, such that samples with higher consumed sugars had higher BC production. The closely equal values of available sugars indicate that all samples reached a limit of maximum sugars extraction from the WS during the 7 days during which the WS was present in the solution throughout SSF.

Average cells concentration and their proliferation rate present a significant indicator to understand the characteristics of SSF. Bacterial cells are the basic BC synthesising unit in the fermentation. Their highest concentration in samples S3 and S4 anticipate highest BC production in these samples which did not occur. Also, samples S3 and S4 had the lowest cellulose yield per sugars consumed. The only difference between sample S1 (highest BC production) and these two samples is the presence of scarification enzymes in S3 and S4. Therefore, the presence of scarification enzymes in SSF is considered to limit BC production. Cellulose polymer is structured in two configurations; cellulose I_α and cellulose I_β. Plant cellulose such as that in WS commonly composed of low degree crystallinity cellulose I_α [32]. Whereas, BC is structured of the two cellulose polymers (cellulose I_α and cellulose I_β), where cellulose I_α form between 20 and 40% of BC by weigh [89]. This explain that enzymes used in SSF samples S3 and S4 depolarized cellulose I_α to glucose, and consequently resulted provided more sugars for cells proliferations instead of BC production (low BC yield based on consumed sugars).

4.2.4 Fermentation inhibitors

The same fermentation inhibitors analyzed in SHF method were also investigated in the SSF method.

Negligible amounts of 5-HMF were measured in all samples, whereas Table 8 shows constant furfural levels during SSF method. Samples S1, S3 and S4 each liberated ~0.3 g/L furfural, while samples S2, S5 and S6 liberated 1.25 to 1.85 g/L. The comparison between furfural levels and total sugars consumption profile (Figure 11) shows that samples with high furfural concentration had lower total sugars consumption at the end of fermentation, which was also correlated with low BC production in the previous sections. Hence furfural plays a role in limiting sugars utilization by *G. Xylinus* to produce BC.

Furfural is formed by hemicelluloses dehydration under elevated acidic conditions as those of samples S2, S5 and S6 [86]. Its concentration over the threshold of 1 g/L has significant effect on inhabiting bacterial growth [19,65,87]. Such effect can be clearly explained when comparing the average cells concentration and proliferation rates in different samples in Table 8.

Samples that were pretreated with 1% acidic solution at 121°C for 30 minutes (S1, S3 and S4) had lower furfural concentration and showed high cells growth rates (Table 8). Whereas the cells growth in the rest of samples were significantly affected by furfural inhibition because of their extreme pretreatment conditions such as; higher acid concentration (2% acidic) in S2, or longer boiling time (90 minutes) and temperature (135°C) in S5 and S6 respectively (Table 4).

4.2.5 Change of bacterium concentration

Figure 12 illustrates the change in cells concentration during the fermentation process. *G. Xylinus* bacteria in all samples experienced between 30 to 35 hours delay phase before starting to grow, except sample S3 which showed a direct cells growth since inoculation at time zero. The exponential growth phase in all samples lasted around 25 hours, and then the cells concentration started to settle constant till the end of fermentation.

Cells concentration reached during the stationary phase and till the end of fermentation can be sorted into three categories. First are samples S3 and S4 that reached highest cells concentration around 12×10^7 cells/mL, whereas sample S1 had the second highest range around 9.5×10^7 cells/mL. The lowest cells concentrations reached were in samples S2, S5 and S6 which reached between 6.5×10^7 and 7×10^7 cells/mL.

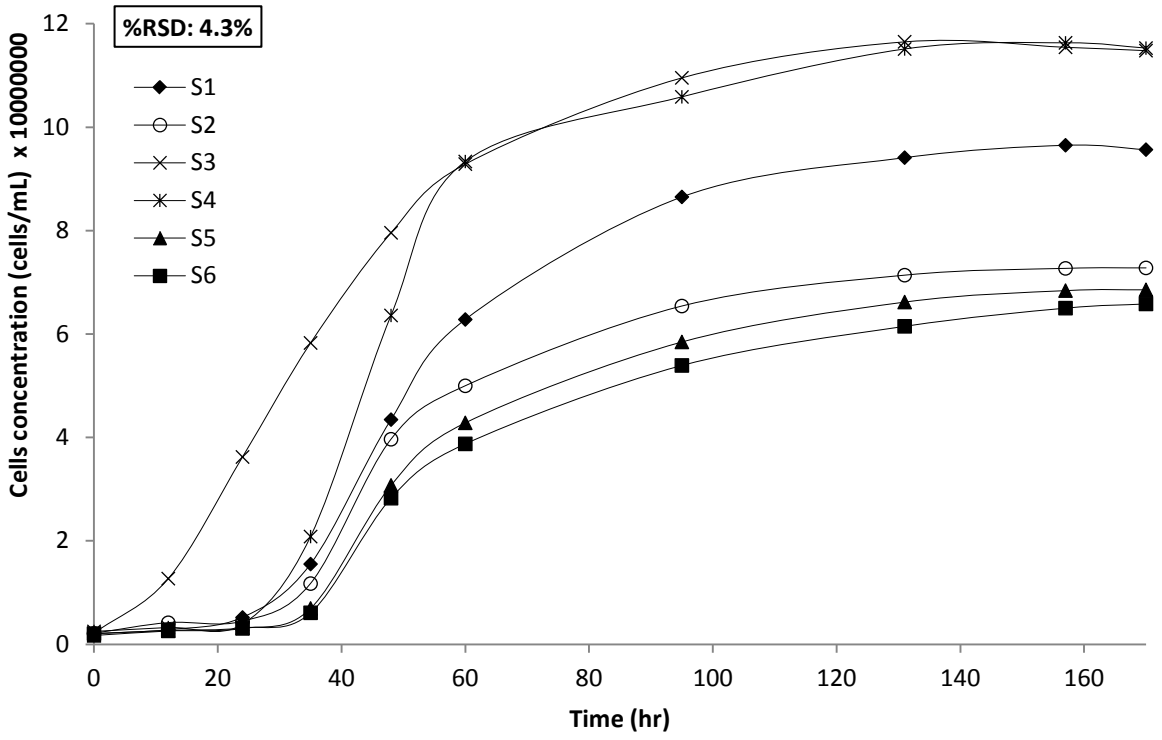

Figure 12. Changes of *G. Xylinus* bacterial concentration during SSF.

This rapid growth of cells concentration in sample S3 without a delay phase is reasoned to high xylose concentration in the sample as its WS was pretreated with enzyme *Xylanase* that break hemicellulose to xylose sugar. Xylose is metabolized by *G. Xylinus* for bacterial cells proliferation and its oxidation produce acetic acid that reduced the medium pH [79,86]. This also explains the pH drop in sample S3 more than the rest of the samples at the end of fermentation in Figure 10. The effect of bacterial inhibition by furfural that was discussed in the previous section can be shown in Figure 12. Samples S2, S5 and S6 reached the lowest bacterial concentration since their furfural concentration exceed the 1 g/L inhibitory threshold.

4.3 Comparison between SHF and SSF

4.3.1 BC production and final pH

The comparison between BC production presented in Figure 7 and Figure 10 shows the highest BC production among all samples was reached in samples S1 (SSF) with 10.8 g/L. This result represents 20% improvement in the SSH sample from its corresponding similar pretreatment sample in SHF (Sample F1). Moreover comparing samples F1, F3 and S1 shows a trend of improving BC production as the exposure time of WS in the fermentation medium increased. The soaking of WS in F3 provided additional sugar resources for fermentation than F1, this was further boosted by maintaining the WS in the medium throughout the fermentation in all the SSF samples.

The use of water pretreatment in samples F8 F9 and F10 showed scarce BC production (3.2 to 3.6 g/L) compared to all the acidic pretreatment samples in SHF (8.1 to 10.5 g/L). This modest efficiency of water hydrolysis in SHF was the reason for not reporting water pretreatment

samples in SSF method. On the other hand, using extreme acidic pretreatment conditions in both SHF (F2, F6 and F7) and SSF (S2, S5 and S6) showed the same effect of decreasing BC production compared with F1 and S2 respectively. As discussed earlier this decrease occur because of the inhibitory effect of furfural on *G. Xylinus* growth.

The use of enzymatic treatment in SHF samples (F4 and F5) increased BC production by ~16% compared to F1 (without enzymes), on the contrary the presence of scarification enzymes in S3 and S4 samples (SSF) decreased BC production compared to enzymatic treatment samples in SHF (F4 and F5), and compared to the control sample in SSF (S1). The enzymes in SHF samples had limited exposure time to WS, in the pretreatment solution, before inoculation which accomplished extra sugars for higher BC. Whereas, in SSF samples S3 and S4 had enzymes present in the fermentation medium all through the reaction, which grated scarification of the plant cellulose in the WS used and part of the amorphous structure of produced BC in the course of fermentation [71,90].

The final pH of SHF fermentation samples was in general lower than their corresponding samples in SSF. This pH drop comes from increasing undesired side products in the form of gluconic acid and acetic acid [80]. This would also explain the lower BC production in SHF samples compared to SSF, and predict that the presence of WS in SSF helped in decreasing undesired acidic side products in the metabolism of *G. Xylinus* [88].

4.3.2 Sugars concentrations

The composition of individual sugars present at the beginning of fermentation in Table 5 and Table 7 did not vary significantly between the two fermentation methods. Glucose was the major sugar component with ~55% of extracted sugars, and then the concentration of xylose came second with ~28% of total sugars. The rest of compositions were equally distributed

between galactose, manose and arbinose. The enzymatic treatment increased glucose and xylose content in the samples it was used in, alike among both SHF and SSF samples.

The total sugars consumption profiles in Figure 8 and Figure 11 shows that SHF samples had a slightly higher sugars consumption rate during the early stage of fermentation (first 60 hours) than their corresponding SSF samples. Nevertheless, the total sugars consumption reached at the end of fermentation were 15 to 30% higher in SSF samples than their corresponding SHF samples. In SSF sugars are extracted from the WS all trough the reaction, while in SHF sugars extraction is only limited in the pretreatment stage. This is the reason behind what was interpreted as slower sugars consumption in SSF than SHF in the first 60 hours of fermentation. In fact sugars would have the same consumption trend if here were no sugars extracted with time in SSF. But, these extracted sugars helped in increasing total sugars available for fermentation in the medium, and thus decreases percentage of consumption till most of the extractable sugars were extracted.

The percentage of consumed sugars that were higher is SSF indicate that this process allowed proper conditions for bacterium activity to consume further sugars than SHF, and which were latter utilized for the yield of higher BC production with the higher consumption of sugars. Similar conclusions can also be drawn from comparing the total sugars available for fermentation in Table 6 and Table 8.

4.3.3 Bacterial cells proliferation

The effect of furfural on inhabiting *G. Xylinus* growth was clearly demonstrated by different samples in both SHF (F3, F6 and F7) and SSF (S2, S5 and S6). Samples that were subjected to sever pretreatment conditions in acidic mediums had elevated furfural concatenations more than the inhibitory threshold concentration of 1 g/L. These samples

experienced a limited growth in bacterial cells that produced BC, which in terms reflected in lower BC production. Whereas, in the samples with lower furfural presence, the average cells concentration and the average proliferation rate was directly related with available sugars for fermentation. SSF samples had a bit higher average cells concentrations and proliferation rates in Table 6 and Table 8, this is because of higher sugars utilized in these samples as discussed before. In addition to that, the effect of xylose high concentration is samples F4 (SHF) and S3 (SSF) reached the same conclusion that xylose enhances sugars proliferation and significantly decreases the delay phase before cells concentration start to grow exponentially after inoculation.

In both fermentation methods, the bacterium undergoes several distinct phases during fermentation as shown in Figure 13

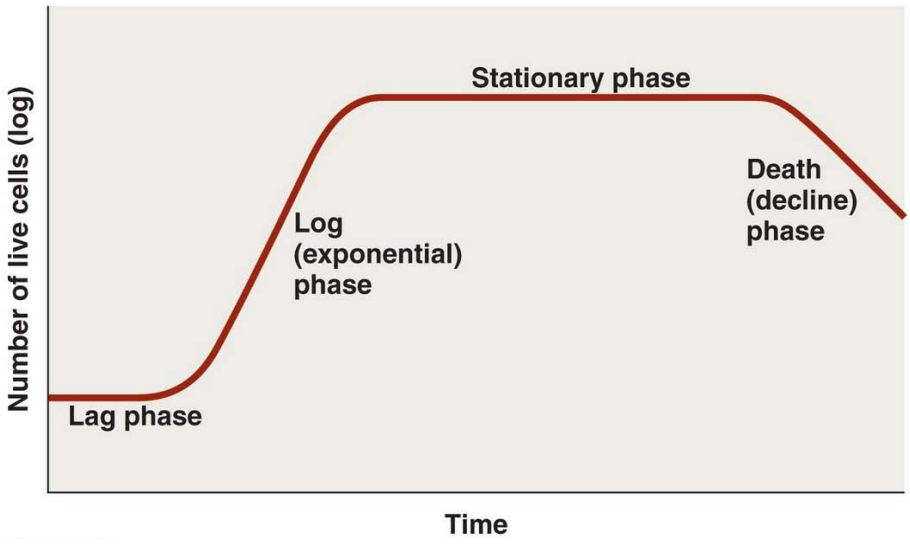

Figure 13. Growth phases of fermentation microorganisms [91]

In typical growth of microorganism there is an initial lag phase after the inoculation where the cells do not start to multiply in numbers. This is followed by the exponential growth phase,

where cell numbers (and dry weight) increases exponentially in a short period of time. Next is a short phase of declining growth increase, followed by stationary phase. There, the cell numbers are the highest. Finally the cell number decline during death phase. The obtained cells proliferation profiles in Figure 9 and Figure 12, in comparison with Figure 13 shows typical growth of cells in both SHF and SSF. It is noticed that bacterial growth went from lag phase, through exponential phase to Stationary phase, but *G. Xylinus* did not undergo a declination phase. This means that the changes in fermentation medium (BC concentration increase and pH drop) dose not cause cells poisoning as it occur in Biomethan production [67].

5. CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The utilization of biocellulose nanofibers in full range of industrial application necessitates decreasing its production cost and improving the fermentation efficiency. In the sake of achieving this goal, WS agricultural residues were examined as a new a new efficient carbon source in the fermentation. A number of trials were performed using two fermentation methods, SHF and SSF. The results revealed that the most suitable WS pretreatment method is using 1% dilute sulphuric acid at 121°C for 30 minutes. The optimal amount of BC produced was 9.7 g/L in SHF and 10.8 g/L in SSF. The soaking of WS in the SHF medium before fermentation helped further sugars extraction that produced higher BC production; consequently SSF had higher BC production since WS was kept in the fermentation medium all the time.

The addition of enzymes during WS treatment increased sugars' concentrations in the fermentation medium and thus increased BC production in SHF method to 10.6 g/L. Nonetheless, enzymes that were present in two SSF samples acted on hydrolyzing the produced BC and the plant cellulose in WS. This eventually led to lower BC production in SSF when using enzymes. Water pretreatment did not provide successful pretreatment condition Moreover the use of pure water (without dilute acids) in the pretreatment of WS proved to produce minor BC quantities, While on the other side, using elevated thermal treatment in the presence of dilute acids increased furfural concentration and inhabited bacterial calls proliferation, that ultimately end with lower BC production than the optimum conditions.

The BC production reached from WS feedstock provides a promising improvement in the fermentation yield and economical cost. The same approach can open the door for next generation of green biomaterials production solely based on renewable agricultural residues.

5.2 Recommendations for Future Work

As it is known that, the production of BC can be increased by increasing the surface area of the air/liquid interface [92]. Rotary disc biological contractor can be used in future to see its effects on producing BC nanofibers. Some important features of this reactor are that it provides more surface area in the form of discs. Medium conditions can be controlled during the fermentation and it is easy to use. Other agricultural wastes sugars compositions for example, rice straw and Soya bean straw can be used to check their suitability for producing BC nanofibers. It is also important to implement pH controlling system that would increase the BC production, or the use of oxygen enrichment in the reaction like utilizing airlift reactors to enhance the bacterial efficiency.

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APPENDICES

Appendix A: List of chemicals and materials

Table A-1 List of materials used and their specification source

Product	Supplier	Catalogue No.
Agar	Sigma-Aldrich (St. Louis, MO)	5038
Ammonium Sulfate	Sigma-Aldrich (St. Louis, MO)	A4418
Arbinose	Sigma-Aldrich (St. Louis, MO)	A3256
Calcium Carbonate	Sigma-Aldrich (St. Louis, MO)	C4830
Calcium Chloride Dihydrate	Sigma-Aldrich (St. Louis, MO)	C3306
Copper Sulfate Pentahydrate	Sigma-Aldrich (St. Louis, MO)	C8027
D-biotin	Sigma-Aldrich (St. Louis, MO)	47868
D-Pantothenic Acid	Sigma-Aldrich (St. Louis, MO)	P2250
Ferrous Sulfate Heptahydrate	Sigma-Aldrich (St. Louis, MO)	215422
Folic Acid	Sigma-Aldrich (St. Louis, MO)	F7876
Fructose	Sigma-Aldrich (St. Louis, MO)	F0127
Furfural	Sigma-Aldrich (St. Louis, MO)	185914
Galactose	Sigma-Aldrich (St. Louis, MO)	G0750

Con't. Table A-1 List of materials used and their specification source

Glucose	Sigma-Aldrich (St. Louis, MO)	G8270
Gluconoacetobacter Xylinum	American Type Culture Collection (Manassas, VA)	ATCC 700178
Hydroxymethyl Furfural	Sigma-Aldrich (St. Louis, MO)	53407
Inositol	Sigma-Aldrich (St. Louis, MO)	I5125
Magnesium Sulfate Heptahydrate	Sigma-Aldrich (St. Louis, MO)	230391
Manganese Sulfate Pentahydrate	Sigma-Aldrich (St. Louis, MO)	229784
Monopotassium Phosphate	Sigma-Aldrich (St. Louis, MO)	P0662
Nicotinic Acid	Sigma-Aldrich (St. Louis, MO)	N4126
Pyridoxine Hydrochloride	Sigma-Aldrich (St. Louis, MO)	P6280
Riboflavin	Sigma-Aldrich (St. Louis, MO)	R9504
Sodium Molybdenum Oxide Dihydrate	Fisher Scientific (Markham, ON)	AA1221436
Thiamine Hydrochloride	Sigma-Aldrich (St. Louis, MO)	T4625
Xylose	Sigma-Aldrich (St. Louis, MO)	X3877
Zinc Slfate Heptahydrate	Sigma-Aldrich (St. Louis, MO)	Z0251
Wheat Straw	local farm from Barrie, ON	N/A
Corn steep liquor	Casco (London, ON)	N/A

Appendix B: HPLC calibration data

B.1 Retention times of standard HPLC sugar solutions

Table B-1. Retention time for each component. Used for their identification in pretreatment extracts from agricultural wastes

Component	Retention time [min]
Mannose	9.38
Glucose	12.92
Xylose	14.02
Glactose	15.73
Fructose	19.43
Arabinose	21.61
Furfural	28.72

B.2 Standard HPLC calibration curves for individual components

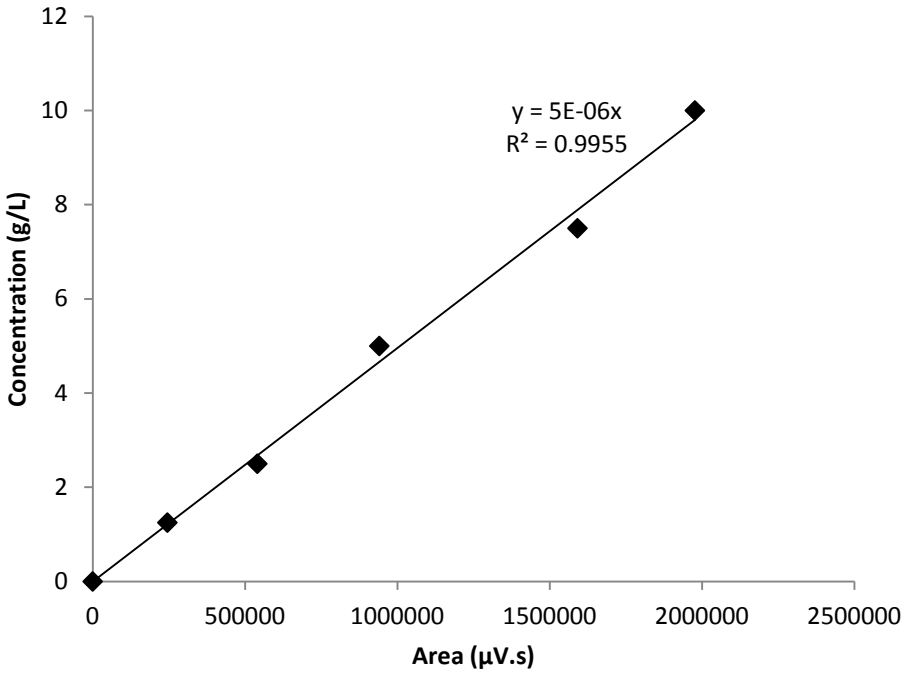

Figure B-1. HPLC calibration curve for mannose

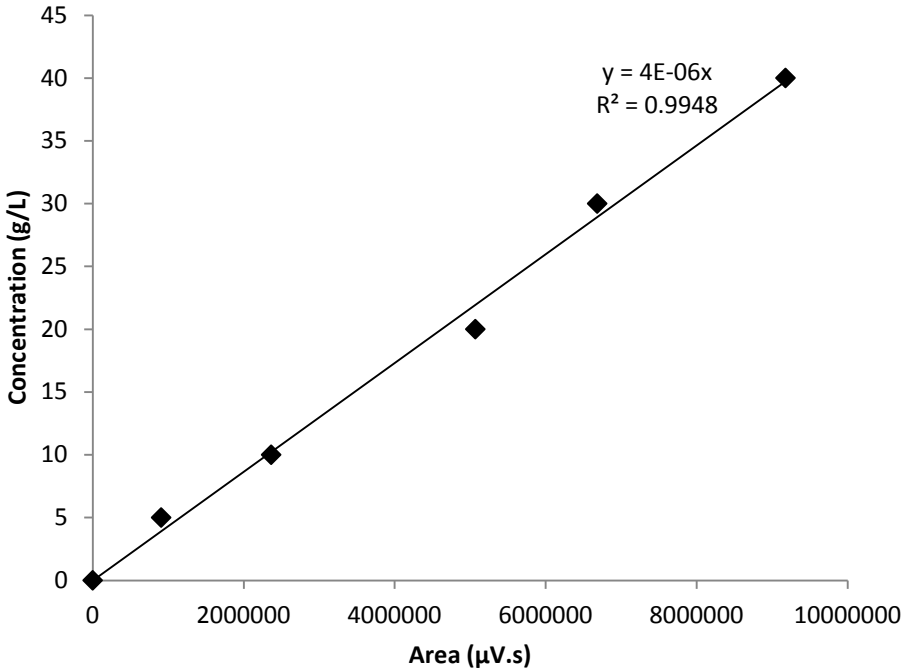

Figure B-2. HPLC calibration curve for glucose

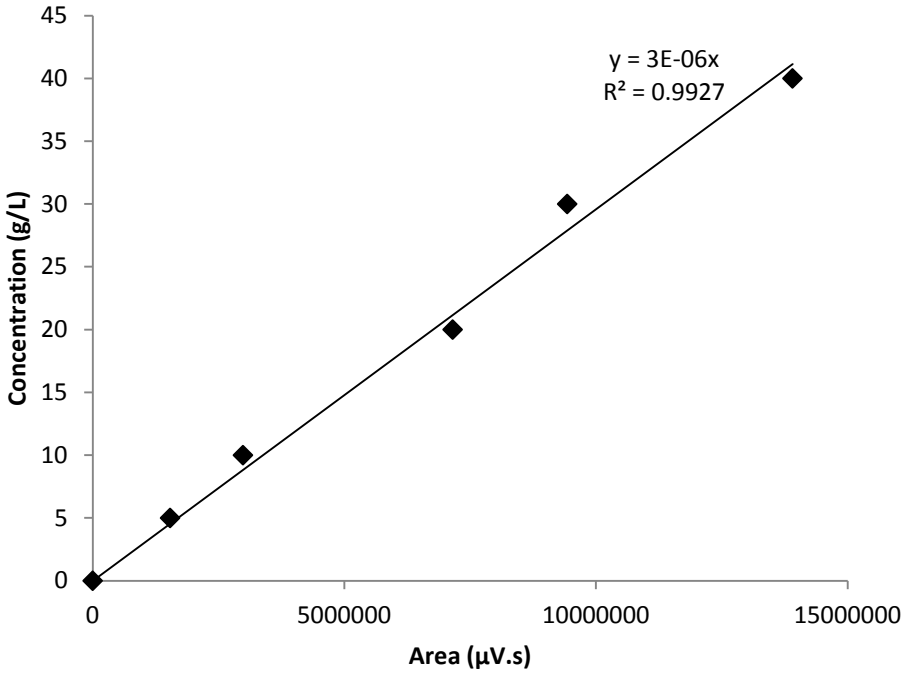

Figure B-3. HPLC calibration curve for xylose

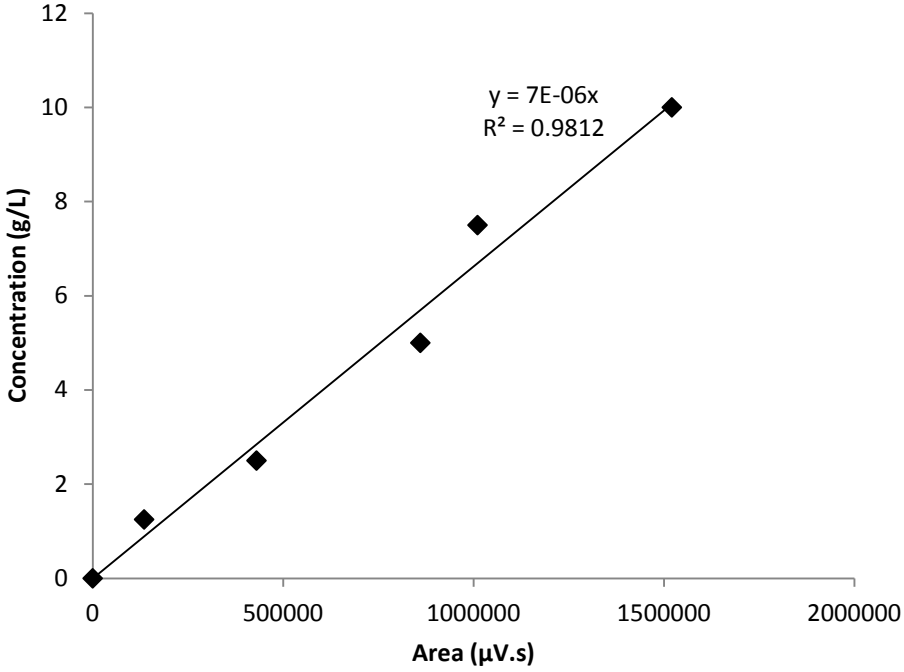

Figure B-4. HPLC calibration curve for galactose

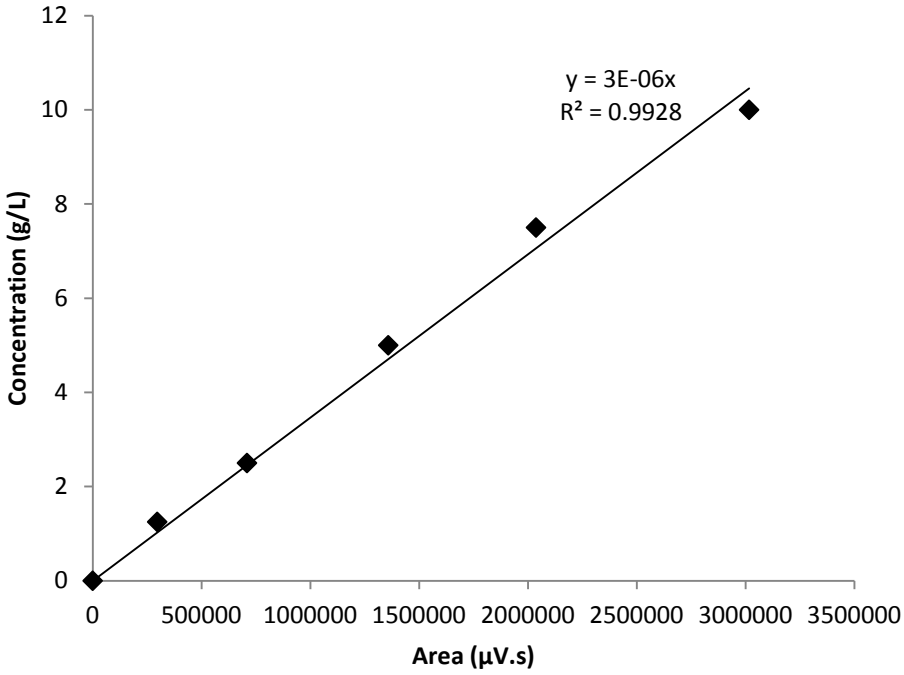

Figure B-5. HPLC calibration curve for fructose

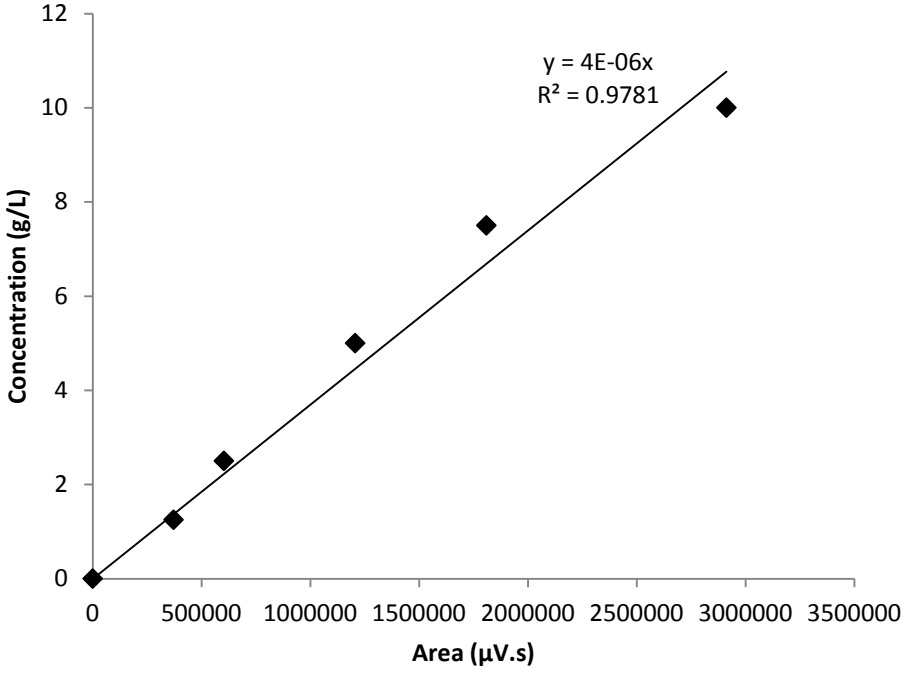

Figure B-6. HPLC calibration curve for arabinose

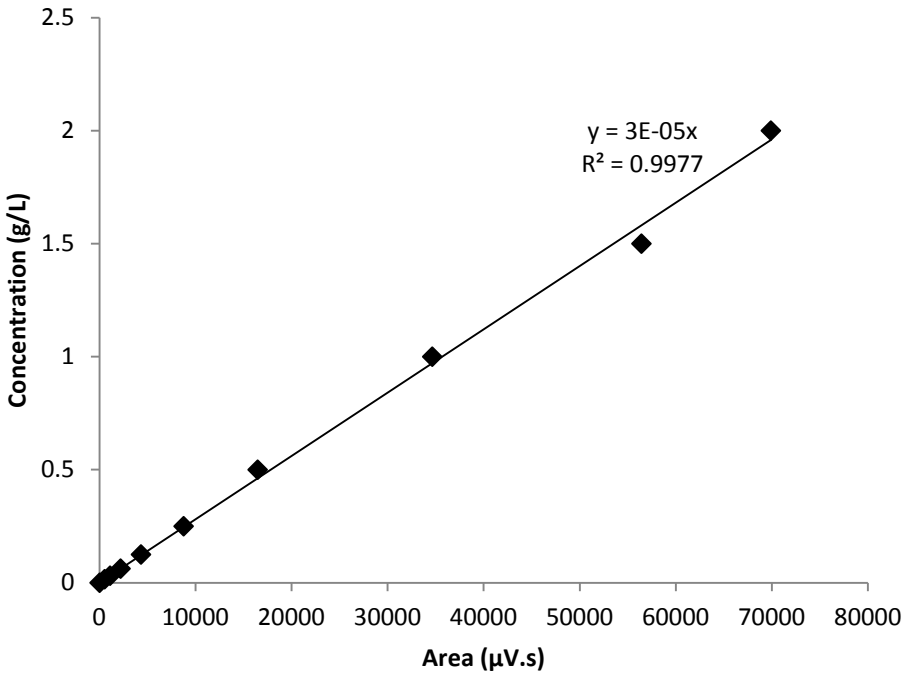

Figure B-7. HPLC calibration curve for furfural

Appendix C: Row data of SHF method

C.1 SHF detailed results

Table C-1. SHF method measurement for BC production triplicates and the final pH.

Sample	Trial 1			Trial 2			Trial 3		
	W_o [a] (g)	W_f [b] (g)	pH	W_o [a] (g)	W_f [b] (g)	pH	W_o [a] (g)	W_f [b] (g)	pH
F1	15.2303	15.3032	2.7	15.5572	15.6261	2.6	15.2879	15.3595	2.8
F2	15.1502	15.2162	3.3	14.9797	15.0468	3.1	14.6749	14.7392	3.2
F3	15.8595	15.9356	2.8	15.3066	15.3822	2.7	15.4613	15.5392	2.9
F4	16.3914	16.4782	1.9	16.4496	16.5358	2.0	15.2144	15.2961	2.0
F5	14.3742	14.4592	2.9	14.8937	14.9777	2.9	14.7783	14.8616	2.9
F6	14.3709	14.4350	3.0	14.1561	14.2240	3.2	14.8134	14.8814	3.1
F7	15.9652	16.0273	3.3	16.2111	16.2719	3.3	16.7533	16.8142	3.0
F8	16.9483	16.9772	3.0	17.2103	17.2392	2.9	16.6863	16.7145	2.8
F9	15.2836	15.3092	2.7	15.3378	15.3643	2.7	16.3492	16.3760	2.6
F10	14.2344	14.2610	3.0	14.5427	14.5708	3.0	15.5167	15.5450	2.8000

[a] *Initial weight of crucible in gram before the 1 ml BC sample*
[b] *Final weight of crucible in gram after reaching a final weight of dried 1 ml BC sample*

C.2 SHF sugars quantification analysis

Table C-2. Sample F1 HPLC analysis areas in triplicates

Time (hr)		Area of HPLC absorbance (μV.s)						
		Glucose	Galactose	Mannose	Xylose	Arbinose	Fructose	H.Furfural
Trial 1	0	4891903.82	285056.58	316979.47	3319256.36	842689.83	541.28	9835.50
	11	2887228.88	202382.93	204246.43	2836124.85	601630.89	340.32	10791.97
	25	2376380.17	183622.41	187963.10	2308800.12	453333.04	269.76	10522.60
	30	2152239.01	169670.07	184620.69	2162578.27	437912.59	231.44	11054.54
	49	1739972.50	126692.22	142774.68	1743678.65	369923.32	182.94	10528.38
	55	1578753.86	113869.38	121963.89	1618477.14	352371.83	163.44	11194.50
	72	1243780.33	91243.61	107688.06	1279945.90	286983.70	139.91	10485.27
	97	1138371.48	77269.79	89768.16	1079472.50	271255.14	112.93	10707.00
	130	932738.19	70638.12	83949.49	996098.23	228374.83	100.01	9884.10
	150	871438.43	74120.54	81158.94	962359.79	240201.95	101.63	10111.39
	157	715383.91	74879.02	81205.95	1023279.47	243077.49	98.53	10310.50
	169	677796.49	67697.95	80596.48	991922.13	221208.71	110.03	10949.57
	175	612859.56	72231.52	79743.73	991458.48	238444.33	98.35	10072.47
Trial 2	0	4658233.39	294023.42	302875.17	3148686.71	869197.77	544.61	9994.75
	11	3076914.66	197019.03	204284.20	2974059.39	585685.45	343.89	10646.73
	25	2527355.45	170946.77	178122.56	2409264.49	420834.25	275.07	9921.96
	30	2379949.27	151650.23	166861.24	2185465.99	386257.51	248.05	9907.20
	49	1673381.84	123885.39	142525.70	1647586.17	365276.38	175.29	10506.41
	55	1569531.25	114032.44	126390.41	1613421.01	352876.40	162.36	11034.22
	72	1353660.31	97082.05	106749.05	1320732.26	305347.04	131.68	11289.12
	97	1172273.74	85303.89	87844.75	1171889.73	249000.56	122.26	9952.63
	130	908056.21	76082.43	79672.03	978733.32	242959.25	105.70	10738.91
	150	855365.27	73831.78	83770.66	1047052.00	239266.19	106.42	10078.18
	157	729390.11	72365.76	83628.31	982077.24	234918.76	101.98	10602.88
	169	646371.33	74009.88	79091.10	970964.76	244471.93	98.45	10081.72
	175	585922.61	72974.68	77018.45	1020895.20	240897.59	102.10	10603.95
Trial 3	0	5025874.32	287002.38	295918.10	3324779.45	848442.03	513.32	10641.56
	11	3091367.26	198382.30	191332.54	2975828.31	589738.07	315.30	9830.30
	25	2458637.51	171798.79	187582.91	2296722.06	421637.65	274.77	10843.34
	30	2169377.15	155075.83	177598.04	2318281.42	404362.84	230.16	10329.42
	49	1788114.38	118661.52	129675.79	1804233.92	353502.79	181.50	10270.30
	55	1488448.53	108525.35	129476.83	1457527.61	335834.57	156.96	10649.06
	72	1324696.63	94337.32	103727.34	1300406.57	296714.18	137.72	11111.64
	97	1052674.10	77608.45	95987.09	1134297.62	253727.02	114.65	10663.88
	130	1010263.40	68639.58	81974.08	1062053.30	224930.64	104.13	10704.04
	150	822603.68	68888.52	82506.20	1040728.05	223246.59	101.80	11137.00
	157	662477.84	69085.21	81892.34	1037262.13	224269.22	109.33	10412.83
	169	645855.84	72111.17	86155.82	1061265.96	235723.09	101.36	10294.95
	175	623494.24	66895.51	86198.22	985418.17	220829.55	99.40	10649.94

Table C-3. Sample F2 HPLC analysis areas in triplicates

Time (hr)		Area of HPLC absorbance (µV.s)						
		Glucose	**Galactose**	**Mannose**	**Xylose**	**Arbinose**	**Fructose**	**H.Furfural**
Trial 1	0	5736085.06	317472.17	405904.30	3977779.82	953450.77	653.38	42002.35
	11	5233961.92	309117.78	266519.66	2740009.31	669317.47	456.83	42574.13
	25	4103351.76	225881.34	257144.28	2734859.99	635917.44	518.94	39878.83
	30	4062326.41	206165.62	254494.86	2652203.69	542851.66	613.02	41970.23
	49	3236570.41	207981.15	215295.47	2547746.81	473997.47	368.45	39421.60
	55	2845507.82	171295.01	194395.94	2265964.18	430232.03	321.84	40804.92
	72	2438389.17	142738.48	162469.81	1885426.44	346503.56	265.63	40022.94
	97	2082213.38	139838.02	147702.89	1659139.94	336494.65	232.63	39889.86
	130	1780453.48	120552.08	140158.01	1503206.05	285658.27	208.89	42095.20
	150	1671206.17	119942.74	137328.77	1561695.46	306516.79	223.30	40866.80
	157	1454424.07	121666.72	141316.84	1540664.66	306053.07	218.50	38785.81
	169	1565558.88	128795.78	131651.42	1495947.15	282550.87	206.81	42962.87
	175	1336843.19	117936.95	141072.63	1454159.52	301589.11	214.87	42226.79
Trial 2	0	5724111.41	299976.58	388917.74	3825545.82	966916.47	652.24	40244.61
	11	5686566.17	285104.91	247021.12	2829611.28	624067.11	442.36	38973.19
	25	4171292.06	238590.00	228306.68	2925535.56	586960.48	475.44	41589.70
	30	4089906.23	233592.66	242262.21	2718088.86	580850.31	616.06	38657.32
	49	3333116.84	205940.01	232300.42	2582031.80	491240.67	375.70	42122.06
	55	2846251.57	180696.14	199908.49	2113212.35	436481.47	327.71	41962.03
	72	2433642.98	153466.51	177810.83	1807086.73	377149.16	260.92	43264.78
	97	2215448.81	131612.42	156322.76	1712009.58	333560.52	250.47	42217.82
	130	1720758.46	124398.97	138105.70	1540564.58	309391.01	209.70	41478.81
	150	1543367.40	131459.39	144225.23	1475308.43	285191.29	211.80	43218.32
	157	1557278.22	125128.93	143226.98	1428193.12	289867.83	221.25	42661.31
	169	1422905.52	116260.12	136386.13	1559754.32	319975.03	213.10	40670.55
	175	1333590.53	118391.90	141598.17	1492462.24	306525.37	204.98	42384.10
Trial 3	0	5595476.95	328497.49	402707.70	4087837.77	896331.87	593.44	41671.57
	11	5348495.85	306260.25	262454.79	2596948.98	645916.57	450.15	42411.03
	25	4569322.29	246436.05	238566.01	2857560.01	601604.99	504.87	42489.13
	30	3778649.01	221452.82	254951.70	2419118.04	578899.13	669.98	43341.12
	49	3263927.52	198420.80	229433.88	2374925.00	503275.30	375.30	42423.63
	55	2715295.44	178705.82	196325.34	2167330.14	415214.20	299.97	41209.91
	72	2261515.24	151480.28	163033.53	1877980.55	369011.72	283.05	40699.08
	97	2152812.98	137647.98	155087.72	1685662.24	324118.52	246.53	41884.27
	130	1726938.13	122585.79	134589.06	1501320.15	298799.94	231.09	40422.62
	150	1642805.4	118735.7	135121.4	1548781.9	310385.9	224.57	39910.67
	157	1561958.3	122862.2	131747	1613090	305596.3	209.93	42548.44
	169	1419904.9	123423.4	147773.8	1507385.3	296157.1	229.77	40362.16
	175	1395391.1	130466.6	131577	1603568	288630.5	229.83	39384.48

Table C-4. Sample F3 HPLC analysis areas in triplicates

Time (hr)		Area of HPLC absorbance (µV.s)						
		Glucose	Galactose	Mannose	Xylose	Arbinose	Fructose	H.Furfural
Trial 1	0	5811868.04	326122.96	410526.45	3846432.08	853625.44	669.22	10275.12
	11	3335222.11	197735.08	267464.70	2886724.83	506574.27	380.11	9423.31
	25	2782834.83	194006.78	235936.62	2850900.50	387660.94	342.51	10533.84
	30	2762163.87	184035.23	242470.06	2826495.18	404407.21	290.37	9636.20
	49	2106417.10	137000.05	188097.15	2078128.45	403454.46	237.15	10148.43
	55	1860511.89	134085.62	163510.38	2027284.66	353518.36	221.92	10851.89
	72	1668987.55	106196.82	142463.65	1686317.21	309264.35	183.12	10907.40
	97	1433395.33	91936.70	116619.59	1452166.32	266251.54	152.28	10444.10
	130	1190114.13	75425.92	113658.79	1201771.38	247351.00	131.07	10748.31
	150	1263094.64	80723.12	106077.68	1234415.22	247461.04	138.45	9736.74
	157	1208580.33	77422.88	111377.46	1216922.02	243713.51	131.18	9597.68
	169	1271802.13	81041.28	111243.80	1317132.04	243482.66	137.25	10154.59
	175	1239654.00	78743.72	105692.67	1271315.26	243346.37	129.05	10476.03
Trial 2	0	5436620.78	333850.93	390209.45	4308166.59	866944.60	617.24	9488.00
	11	3608199.95	216856.19	249311.48	2771637.59	513960.43	396.66	10259.03
	25	2944716.50	205366.89	244784.95	2664449.79	426111.30	327.27	10164.18
	30	2649668.15	176539.95	238998.43	2597061.52	382107.59	308.96	10374.95
	49	2035164.99	132365.86	182910.31	2165314.59	395707.08	230.28	9760.18
	55	1863800.57	117765.07	173641.13	1945320.26	375980.05	202.78	10938.08
	72	1689499.18	106457.59	143580.25	1689844.64	318083.77	178.62	10779.04
	97	1416833.10	90874.42	123448.28	1387527.83	262455.52	144.62	9670.90
	130	1232189.44	79066.81	101038.33	1292394.01	243113.54	135.34	9433.78
	150	1303067.59	83858.38	114669.07	1358175.41	231795.98	131.57	10677.90
	157	1288160.68	82520.88	110943.56	1291969.64	224440.12	136.16	10740.15
	169	1225155.22	78068.87	104545.15	1192871.92	245830.70	131.14	9901.56
	175	1266722.73	80463.15	105328.73	1212370.76	235483.98	130.14	10345.49
Trial 3	0	5922679.31	303556.63	411847.84	4170882.70	923870.59	652.56	10414.47
	11	3480884.42	211919.28	267222.19	2762655.17	541022.21	402.66	10536.91
	25	2919358.00	203668.72	265574.01	2771348.20	393630.94	319.75	9524.16
	30	2746850.57	183014.95	230655.87	2766017.90	401212.14	300.22	10211.84
	49	2258307.82	146878.93	184725.71	2194902.65	366442.65	232.23	10329.85
	55	2058823.33	125805.63	169386.22	1958249.52	347972.98	200.38	10271.96
	72	1565487.25	100655.51	137557.47	1648421.63	297165.83	170.49	10536.38
	97	1323228.86	84870.72	122427.49	1390573.62	264852.91	152.89	10146.06
	130	1316337.50	85407.40	110475.85	1301541.41	223417.67	133.40	10083.48
	150	1198478.34	76012.92	105880.62	1231496.17	241247.70	139.78	9850.63
	157	1247312.06	79904.08	103515.55	1307865.16	251898.09	132.48	9927.49
	169	1238271.97	78904.69	108107.21	1270729.84	229868.36	131.43	10209.50
	175	1199441.33	76189.39	110102.77	1272415.80	239108.62	140.62	9444.37

Table C-5. Sample F4 HPLC analysis areas in triplicates

Time (hr)		Area of HPLC absorbance (µV.s)						
		Glucose	Galactose	Mannose	Xylose	Arbinose	Fructose	H.Furfural
Trial 1	0	7024446.10	407097.81	443446.26	4958054.41	962150.57	759.64	10471.41
	12	4517044.74	318561.67	297552.41	3269890.47	559136.09	508.62	10041.98
	24	3571102.37	232444.75	277404.80	3435772.94	479046.87	387.44	10042.88
	33	2955992.01	237715.57	262173.42	3320015.36	435543.81	351.83	9974.71
	52	2348834.45	178707.42	190151.20	2614215.96	402999.97	268.06	10225.94
	77	1912993.90	144202.45	159958.39	1905868.11	347770.96	192.29	9136.35
	95	1713460.52	119621.79	134988.80	1770796.47	313979.94	173.64	10151.76
	124	1440001.29	107205.68	126092.66	1743534.40	300759.75	153.98	10722.43
	151	1363860.61	100913.56	109334.92	1443290.60	244686.63	147.55	10630.50
	160	1302752.66	102300.02	117986.29	1531021.59	256972.69	149.74	10478.77
Trial 2	0	6715701.37	435967.61	445599.90	4940981.91	1002516.79	813.51	9857.46
	12	4033175.89	298896.99	318297.88	3453862.76	607129.78	473.92	9939.00
	24	3616473.57	264281.69	262523.04	3442496.39	428249.02	425.50	10023.41
	33	3290332.46	226348.58	246426.30	3361642.32	436396.12	324.14	10782.66
	52	2408381.46	180618.45	198477.96	2601933.68	421831.36	268.29	10637.89
	77	1750464.97	131062.37	151624.65	2060657.56	316412.23	192.94	9631.38
	95	1706785.36	122547.64	143313.57	1887396.57	315201.66	177.89	10537.01
	124	1569714.20	114017.72	124155.46	1615280.20	270020.37	165.81	10536.79
	151	1311682.96	98994.04	116862.55	1504174.56	255258.60	144.74	10713.39
	160	1412626.94	98547.88	112734.51	1438829.71	250569.58	144.24	10579.82
Trial 3	0	7146338.23	437116.36	458268.79	5164932.92	1016215.74	815.66	10515.96
	12	4167997.48	302885.15	310902.15	3567443.26	602348.04	476.72	10912.99
	24	3362230.27	251353.52	274647.73	3212451.17	466039.55	406.44	10831.70
	33	3067343.05	238266.66	251029.65	3124766.83	407233.90	363.50	10143.22
	52	2403864.73	167078.97	202759.61	2460272.98	436190.11	253.25	10055.31
	77	1826703.19	130752.45	150546.35	2061961.73	333591.25	212.48	9688.20
	95	1592510.88	129657.84	145076.80	1827545.70	298472.35	188.21	10252.27
	124	1538423.01	115554.01	134372.25	1629363.16	276112.35	169.96	9686.89
	151	1388241.97	100864.24	118579.30	1496619.61	260628.25	147.48	9607.74
	160	1334041.19	99602.95	112981.97	1487853.47	258362.20	145.79	9892.85

Table C-6. Sample F5 HPLC analysis areas in triplicates

Time (hr)		Area of HPLC absorbance (µV.s)						
		Glucose	**Galactose**	**Mannose**	**Xylose**	**Arbinose**	**Fructose**	**H.Furfural**
Trial 1	0	7391236.15	305242.05	297704.19	3459572.03	875321.46	514.57	9602.59
	12	4439946.85	230816.06	205337.72	2445124.52	521378.18	523.01	10271.31
	24	3234354.41	156326.92	177105.96	2182880.32	424287.49	385.86	9637.29
	33	2894584.98	147004.21	159495.65	2021436.21	371381.51	327.98	9634.90
	52	2342208.64	108051.01	113475.55	1613682.21	341814.57	260.56	10142.71
	77	1841799.73	87199.09	100546.81	1374889.54	316528.75	219.70	10291.80
	95	1551339.01	74113.94	81848.33	1160359.58	256469.17	177.03	9830.80
	124	1385183.50	62735.89	72660.90	1087849.25	225970.32	159.10	9630.37
	151	1326018.69	59814.43	69033.37	925104.76	206258.48	147.87	10314.21
	160	1325979.71	58220.43	66134.72	874950.26	204652.65	138.73	9676.97
Trial 2	0	7418298.54	297898.21	310640.05	3075392.25	871803.80	526.96	9820.10
	12	4254140.92	230413.54	194021.75	2440020.51	501424.46	516.44	10043.75
	24	3513768.40	164840.32	175646.00	2015443.21	408424.39	408.38	10196.02
	33	2951376.71	151866.12	147700.32	2001605.45	379793.74	341.04	10018.31
	52	2328301.43	109842.39	119153.31	1509138.85	359800.29	253.31	9860.44
	77	1884279.02	88231.23	97748.65	1346995.11	296022.25	203.42	9529.03
	95	1613049.98	69754.38	85704.97	1066939.02	269110.05	175.29	9733.89
	124	1487275.04	63260.45	72935.19	983045.15	246713.68	170.90	10589.57
	151	1318489.79	55960.10	69962.19	930540.70	218417.83	145.47	10146.68
	160	1321058.33	56889.93	64811.09	900096.24	212078.63	145.90	10177.21
Trial 3	0	6751830.98	271612.47	309353.44	3257755.47	818636.09	557.35	10372.92
	12	4404981.07	209639.80	212468.78	2385187.42	535803.77	499.79	9620.23
	24	3482524.68	165726.34	158917.81	2066394.16	384592.81	405.16	10111.97
	33	3121154.11	149176.64	150401.21	1890703.03	346071.68	350.47	10295.50
	52	2208458.11	99447.86	118906.30	1552711.70	370453.08	255.75	9955.66
	77	1998892.95	84465.68	98692.54	1283270.16	304569.20	216.56	10144.67
	95	1641886.02	72953.22	81984.30	1137187.24	258358.25	187.41	10407.26
	124	1487074.50	69009.21	80712.51	992060.45	242086.97	159.76	9755.07
	151	1249952.59	57583.89	63715.05	877313.41	216807.66	136.45	9517.48
	160	1232137.28	56901.93	70622.99	969149.35	228620.44	145.14	10124.04

Table C-7. Sample F6 HPLC analysis areas in triplicates

Time (hr)		Area of HPLC absorbance (µV.s)						
		Glucose	**Galactose**	**Mannose**	**Xylose**	**Arbinose**	**Fructose**	**H.Furfural**
Trial 1	0	5510191.06	300407.69	381128.65	3639408.46	894633.32	574.30	53187.92
	12	3775020.39	234051.90	256996.56	2607267.67	811927.51	392.52	53966.45
	24	3640787.87	209372.70	205319.66	2339884.96	603614.71	390.13	51542.47
	33	3336241.94	196561.84	208241.79	2156816.15	548621.98	458.80	53190.23
	52	3204014.59	179905.13	191454.90	2104678.65	522774.72	350.29	53351.26
	77	2915331.48	146601.47	178305.35	2047390.04	439971.80	304.79	49006.13
	95	2275201.23	131778.77	138180.87	1666682.72	363552.69	264.45	53522.75
	124	2089151.82	113559.55	124729.43	1468860.31	350237.10	252.55	50563.51
	151	1917443.37	101823.64	120577.68	1345569.14	305208.91	216.73	50565.08
	160	1996334.27	101177.15	119225.13	1382840.47	292774.26	217.12	49981.34
Trial 2	0	5206350.96	288172.87	350191.49	3587300.26	963219.60	611.05	51021.71
	12	3871105.17	233821.47	251782.49	2746969.93	786949.28	389.11	53389.56
	24	3426628.06	220947.77	231121.82	2520849.38	544465.76	381.88	54771.21
	33	3401698.98	201014.20	201297.08	2025866.60	547007.96	447.07	54395.05
	52	3278947.29	171062.32	186106.23	2112519.28	478404.98	357.45	50728.91
	77	2740329.16	155489.02	162908.38	1958996.30	440829.85	327.76	53719.03
	95	2466745.72	123179.29	148263.52	1776101.12	391454.57	262.13	49413.54
	124	2202924.82	120143.26	139375.69	1595754.29	337142.56	250.47	53514.37
	151	1980859.45	105875.62	119394.78	1371969.19	312122.25	212.92	52577.27
	160	2035419.66	104719.41	114424.55	1422054.09	304420.57	223.60	51731.21
Trial 3	0	5466657.17	298742.25	372973.81	3462080.74	948076.93	593.77	52893.05
	12	3605283.87	213589.43	239870.92	2750081.99	753627.10	377.77	49772.08
	24	3583768.53	208010.92	214306.49	2388571.30	574129.44	417.40	50828.00
	33	3597381.07	183166.06	202058.50	2187966.93	515649.72	423.46	49565.29
	52	3204690.14	178965.39	206142.84	1963011.77	507422.71	381.71	53072.58
	77	2699001.43	152795.19	163500.24	1966163.36	408853.29	306.99	54430.74
	95	2377352.69	131999.07	143311.58	1638565.92	347267.68	273.01	54228.71
	124	2214765.54	119152.89	128450.86	1581565.17	317470.28	226.61	53091.58
	151	1945779.38	108805.00	112779.51	1450891.47	284408.78	230.01	54031.99
	160	1848695.78	112270.57	120854.29	1396565.24	309862.61	218.95	55461.47

Table C-8. Sample F7 HPLC analysis areas in triplicates

Time (hr)		Area of HPLC absorbance (µV.s)						
		Glucose	**Galactose**	**Mannose**	**Xylose**	**Arbinose**	**Fructose**	**H.Furfural**
Trial 1	0	5326881.55	308904.82	361370.22	3560019.86	830803.91	582.06	62112.37
	12	3858065.33	275540.59	294320.78	3239100.15	655629.42	498.77	69299.23
	24	3414477.07	248570.01	233978.06	3030499.61	564783.63	472.20	62559.08
	33	3723729.10	210257.07	230329.54	2427189.56	572527.33	398.90	61203.18
	52	3322734.34	187799.54	207043.00	2404288.46	494261.87	375.55	59512.57
	77	2890574.58	164463.29	176983.94	1967666.63	400029.04	304.28	60587.13
	95	2451641.96	136888.87	153903.38	1661845.97	347688.27	265.55	59233.04
	124	2116143.94	128886.16	140011.60	1593183.84	329869.88	255.27	61124.73
	151	1947360.73	116002.58	121247.29	1437072.75	277842.57	215.32	61310.61
	160	2067513.34	110764.36	131409.55	1457525.20	298343.84	217.24	56316.02
Trial 2	0	4933947.54	305758.98	343257.91	3429446.14	868998.93	565.27	61479.83
	12	3818422.65	251011.18	272801.93	3001632.69	732823.88	535.68	63890.81
	24	3417219.73	228515.64	252936.06	2754585.90	608006.00	430.22	58086.11
	33	3779065.40	197260.61	230811.34	2375356.79	545929.43	381.60	57420.08
	52	3211329.75	197230.57	200138.27	2222212.83	493829.85	373.13	62501.21
	77	2848590.83	158230.47	168136.85	2070987.81	427473.26	314.82	58291.01
	95	2352520.51	142656.57	145174.94	1688754.49	363273.54	255.44	61728.77
	124	2271427.04	121217.48	130193.99	1644845.41	331140.60	242.47	56779.45
	151	1993424.80	116344.86	118923.38	1449326.32	291694.76	229.88	61491.51
	160	2008290.87	114968.91	118286.45	1399416.94	302697.74	216.15	60412.75
Trial 3	0	5177915.63	283171.43	362937.41	3466162.47	884078.29	561.83	56938.08
	12	4216316.23	253397.83	291455.99	3253788.18	714059.65	513.16	65405.43
	24	3583480.68	233222.61	253054.45	2748328.06	623564.53	446.92	59907.20
	33	3628910.43	212798.71	225153.49	2414458.28	572912.40	378.91	61943.02
	52	3222862.44	184780.86	200895.30	2361589.36	466545.22	360.78	58555.97
	77	2676998.16	167487.38	182223.99	2020054.27	415930.89	340.42	61701.19
	95	2368156.17	137798.68	150694.85	1798041.27	351800.88	288.62	59626.72
	124	2168017.44	130690.62	141002.58	1467803.51	307803.46	241.88	62688.88
	151	1947250.43	109352.52	129485.70	1333442.72	299855.89	224.46	57795.87
	160	1849208.24	117954.44	122273.97	1394881.66	273470.39	236.27	63868.92

Table C-9. Sample F8 HPLC analysis areas in triplicates

Time (hr)		Area of HPLC absorbance (µV.s)						
		Glucose	Galactose	Mannose	Xylose	Arbinose	Fructose	H.Furfural
Trial 1	0	3385804.65	6383.37	8373.34	2063612.88	451784.38	396.38	6679.10
	11	2060926.52	4095.56	5239.61	1751599.68	290420.11	236.87	6702.46
	25	1631701.60	3519.98	4769.34	1452857.33	249912.26	171.63	6784.76
	30	1581050.70	3513.35	4299.77	1294187.45	228645.60	174.18	6443.88
	49	1160873.08	2407.91	3540.64	1059030.10	178748.57	130.67	6249.33
	55	923229.34	2256.66	3107.44	888243.36	163054.17	111.64	6456.72
	72	798255.94	1904.22	2431.18	753592.73	128934.14	91.62	6616.66
	97	777828.46	1800.37	2357.58	627386.44	127218.56	80.39	6424.07
	130	678937.04	1464.25	1948.60	572705.27	110944.62	70.74	6565.39
	150	631012.64	1457.31	2017.77	581976.33	117122.34	69.14	6933.28
	157	666952.94	1530.96	1945.33	574876.60	105778.61	73.78	6872.66
	169	687413.11	1543.87	2096.96	605898.79	109672.24	74.87	6774.62
	175	661036.79	1503.02	1944.97	574056.88	112193.40	73.56	6782.62
Trial 2	0	3526235.31	5962.69	7747.21	2316857.79	419627.10	405.19	6545.74
	11	1975995.15	4127.49	5204.95	1904774.37	285668.60	238.72	6574.97
	25	1559983.60	3769.61	4792.50	1447854.58	260397.12	183.81	6386.96
	30	1411687.50	3296.48	4599.55	1367001.70	248271.50	162.30	6430.55
	49	1175622.08	2737.72	3350.70	1061102.51	191885.83	120.86	7047.97
	55	1038559.68	2148.75	2920.80	899380.32	163373.25	102.86	6979.71
	72	818493.11	1924.59	2537.64	750052.15	134958.98	91.70	6883.21
	97	706412.49	1636.32	2234.75	678341.07	115747.19	80.66	6772.15
	130	676292.09	1500.07	2068.77	610415.70	111376.32	72.48	6814.54
	150	687853.20	1549.91	1986.77	607468.33	105151.77	74.45	6813.24
	157	641437.10	1501.84	2079.26	599127.27	110348.90	72.38	6926.25
	169	622561.57	1487.25	2003.62	569414.04	106939.48	72.13	6839.95
	175	630387.84	1535.84	2102.08	572080.18	110795.30	75.17	6563.48
Trial 3	0	3176369.28	6486.65	8216.65	2143439.01	463256.96	367.86	6741.63
	11	1966709.53	3701.53	5031.83	1788223.68	265383.51	214.08	6699.18
	25	1660015.81	3779.54	4928.76	1513478.88	263955.33	184.29	6813.63
	30	1458877.59	3340.46	4492.68	1411955.64	246316.12	163.26	7114.03
	49	1092974.16	2603.79	3505.45	990194.21	185291.07	128.26	6700.34
	55	977974.58	2261.89	2975.36	836006.19	152455.81	115.35	6565.80
	72	816958.09	1780.32	2592.38	709216.02	139552.86	86.56	6506.25
	97	731352.69	1632.45	2218.86	678770.39	122031.47	88.83	6812.10
	130	631012.28	1587.10	2094.23	591058.93	105465.03	76.68	6630.40
	150	674141.32	1570.79	2150.26	594664.25	107524.11	76.31	6263.69
	157	678875.87	1530.21	2109.81	596118.06	112670.21	73.74	6211.45
	169	663195.73	1503.17	2008.02	588815.09	110265.26	72.90	6395.82
	175	661609.03	1454.27	1978.75	598009.83	101144.77	71.17	6664.50

Table C-10. Sample F9 HPLC analysis areas in triplicates

Time (hr)		Area of HPLC absorbance (µV.s)						
		Glucose	**Galactose**	**Mannose**	**Xylose**	**Arbinose**	**Fructose**	**H.Furfural**
Trial 1	0	3448636.83	4966.03	6492.43	2580394.71	615423.35	402.68	7643.24
	12	2131811.29	3172.65	3900.18	1724317.12	362573.35	245.07	7673.97
	24	1790600.03	2888.05	4028.17	1941894.19	349328.79	208.40	7295.15
	33	1706115.43	2878.61	3745.19	1667807.11	341957.67	183.58	7726.83
	52	1430006.92	2197.61	2716.87	1346024.33	278503.60	149.74	7656.78
	77	1198302.45	1889.69	2496.97	1129440.85	247039.02	129.61	7769.59
	95	1027663.24	1527.31	1994.53	961003.30	204289.95	108.12	7421.75
	124	946045.85	1415.29	1916.68	865784.70	182788.25	92.11	7570.03
	151	876645.41	1246.24	1602.31	745637.77	172015.33	84.57	7449.15
	160	952861.56	1182.87	1687.69	795101.81	165273.92	85.66	7294.07
Trial 2	0	3256650.76	4996.61	6412.86	2409279.37	589743.30	390.13	7690.32
	12	2212978.49	3080.08	4146.72	1775512.65	381704.86	247.60	7450.09
	24	1884818.49	3001.81	3792.42	1970514.65	337636.23	198.01	7582.51
	33	1777694.08	2678.01	3536.50	1726214.25	356719.21	184.22	7188.38
	52	1313929.69	2108.10	2925.16	1271802.55	296324.32	147.59	7344.94
	77	1148920.07	1904.43	2456.12	1107957.21	245404.69	117.60	7830.18
	95	936016.67	1498.03	2124.95	902666.11	215584.03	97.95	8036.38
	124	955418.73	1361.13	1874.23	838067.10	195498.02	90.34	7280.32
	151	941084.11	1190.47	1762.81	787664.31	166374.72	87.34	7204.60
	160	867720.42	1291.57	1659.79	771423.90	174891.02	85.82	7706.13
Trial 3	0	3423213.62	4709.49	6162.71	2549167.55	577980.00	396.60	7248.40
	12	2154634.89	3092.69	4157.10	1713811.98	383360.23	226.97	7480.58
	24	1940623.22	3057.01	3967.61	1824918.87	377737.17	203.28	7721.94
	33	1801985.48	2864.80	3674.91	1690756.40	385136.81	191.91	7689.76
	52	1399409.68	2185.42	2879.17	1335389.91	287946.55	142.44	7614.33
	77	1143521.05	1707.88	2281.71	1128888.78	237242.52	122.60	7022.09
	95	982208.19	1631.08	1977.12	947761.46	202961.24	103.77	7169.16
	124	893628.54	1454.45	1746.50	850452.06	188018.45	97.41	7779.48
	151	898246.11	1349.71	1598.67	739983.80	169102.66	77.96	7978.88
	160	876603.15	1318.85	1599.51	712051.18	169305.53	78.40	7632.37

Table C-11. Sample F10 HPLC analysis areas in triplicates

Time (hr)		Area of HPLC absorbance (µV.s)						
		Glucose	**Galactose**	**Mannose**	**Xylose**	**Arbinose**	**Fructose**	**H.Furfural**
Trial 1	0	3540302.14	6175.81	7973.18	2502546.82	519746.38	406.54	10209.91
	12	2352506.71	4161.79	5441.53	1844472.20	403149.12	238.19	10304.03
	24	1896585.79	3962.48	5136.50	1611504.50	342187.66	217.81	10244.43
	33	1932435.24	4061.00	4631.82	1635375.19	325428.26	211.41	10221.67
	52	1556208.05	3184.76	3931.72	1282073.41	276257.40	157.60	9431.08
	77	1264156.53	2664.88	3317.23	1113085.92	232442.09	137.82	10098.40
	95	1027831.78	2282.97	2822.27	910804.79	181890.19	114.74	10185.67
	124	1000450.28	2059.77	2391.08	858778.36	169099.58	108.90	10089.22
	151	891185.91	1873.20	2319.92	752875.90	158884.34	90.04	10236.18
	160	906427.35	1796.65	2191.61	768623.60	154460.19	90.33	9775.54
Trial 2	0	3562948.30	6080.06	8250.09	2465423.41	499862.28	394.76	9953.96
	12	2137966.24	3929.99	5776.64	1984478.87	425601.68	253.73	9797.96
	24	2060565.46	3591.36	5449.95	1804071.62	325962.07	219.29	9596.12
	33	1919404.08	3939.03	5057.48	1627292.50	348502.68	209.80	10078.34
	52	1460336.37	3120.42	3689.64	1293395.43	257470.54	166.79	10112.41
	77	1200963.77	2537.90	3107.69	1126232.88	221662.87	140.08	9617.20
	95	1070953.61	2123.88	2665.01	942701.47	189741.98	115.14	9475.87
	124	919241.24	1975.88	2574.39	811424.04	179919.82	107.34	9678.32
	151	854459.88	1887.81	2281.96	738555.09	157833.21	97.91	10316.00
	160	825592.54	1762.21	2334.91	721964.71	162999.45	97.81	10585.10
Trial 3	0	3423482.77	5919.84	7611.73	2305709.41	529037.75	398.10	9592.47
	12	2133258.97	3848.64	5994.03	1904327.63	426891.64	257.72	9666.98
	24	1875518.19	3963.16	5388.56	1754473.61	336655.21	202.60	9934.99
	33	1785369.66	3637.84	5164.30	1778511.06	335831.50	198.48	9476.46
	52	1435700.62	2886.40	3923.84	1335238.96	267030.53	165.36	10243.87
	77	1301585.02	2659.35	3374.88	1088137.02	221490.51	131.90	10077.46
	95	1085587.71	2272.02	2769.32	923337.61	204857.54	119.95	10136.80
	124	979044.08	2048.35	2533.92	851993.48	174833.07	103.59	10033.29
	151	850444.59	1692.98	2119.92	752507.90	152583.67	91.92	9251.36
	160	866334.74	1918.72	2172.47	758032.59	153455.57	91.73	9442.83

C.3 SHF cells counting

Table C-12. Sample F1 hemocytometer cell counting

Dilution	Trial 1 # of cells count (cells)			Trial 2 # of cells count (cells)			Trial 3 # of cells count (cells)		
	Quadrant 1	Quadrant 2	Quadrant 3	Quadrant 1	Quadrant 2	Quadrant 3	Quadrant 1	Quadrant 2	Quadrant 3
150	56	58	53	59	57	59	54	55	57
150	111	103	107	106	112	112	116	118	115
250	72	72	72	71	70	76	70	71	66
500	99	96	97	97	89	91	86	96	93
3000	62	66	64	64	64	67	61	57	56
3000	74	71	79	70	74	72	76	75	69
3000	93	88	90	93	97	97	95	96	94
3000	100	98	91	97	100	97	101	100	111
3000	98	105	101	109	106	107	109	103	107
3000	110	111	102	103	108	108	115	108	118
3000	114	116	107	110	114	117	111	105	111
3000	119	117	113	114	114	120	106	108	106
3000	119	118	109	103	106	114	119	117	116

Table C-13. Sample F2 hemocytometer cell counting

Dilution	Trial 1 # of cells count (cells)			Trial 2 # of cells count (cells)			Trial 3 # of cells count (cells)		
	Quadrant 1	Quadrant 2	Quadrant 3	Quadrant 1	Quadrant 2	Quadrant 3	Quadrant 1	Quadrant 2	Quadrant 3
150	46	47	43	48	46	49	45	45	47
150	69	64	66	66	70	70	72	74	71
250	50	50	50	49	49	52	49	49	46
500	33	32	33	33	30	31	29	32	31
3000	41	43	42	42	42	44	40	38	37
3000	51	49	54	48	51	50	52	51	47
3000	71	67	68	71	74	74	72	73	71
3000	83	82	76	81	84	81	84	83	92
3000	77	83	80	86	84	85	86	82	85
3000	84	85	78	78	83	82	88	82	90
3000	85	86	80	82	85	87	82	78	83
3000	88	86	83	84	84	88	78	79	78
3000	86	85	79	75	77	83	86	85	84

Table C-14. Sample F3 hemocytometer cell counting

Dilution	Trial 1 # of cells count (cells)			Trial 2 # of cells count (cells)			Trial 3 # of cells count (cells)		
	Quadrant 1	Quadrant 2	Quadrant 3	Quadrant 1	Quadrant 2	Quadrant 3	Quadrant 1	Quadrant 2	Quadrant 3
150	56	58	57	56	56	55	52	51	53
150	69	76	73	75	73	81	81	76	72
250	85	87	79	77	82	83	88	81	88
500	42	44	45	44	43	41	47	45	47
3000	50	53	52	46	49	46	53	48	52
3000	60	58	61	62	66	64	65	64	62
3000	85	92	91	96	89	94	94	94	90
3000	122	122	114	116	121	123	115	110	115
3000	152	136	141	131	138	136	127	136	133
3000	150	143	151	140	134	134	140	154	146
3000	155	146	149	149	150	145	134	140	143
3000	148	141	151	138	145	146	151	150	140
3000	142	148	151	157	141	151	134	142	130

Table C-15. Sample F4 hemocytometer cell counting

Dilution	Trial 1 # of cells count (cells)			Trial 2 # of cells count (cells)			Trial 3 # of cells count (cells)		
	Quadrant 1	Quadrant 2	Quadrant 3	Quadrant 1	Quadrant 2	Quadrant 3	Quadrant 1	Quadrant 2	Quadrant 3
150	59	61	56	57	58	61	57	54	56
500	101	103	103	112	111	106	104	103	108
3000	41	41	38	42	45	44	41	39	44
3000	67	67	63	62	63	61	64	63	68
3000	97	91	97	93	98	97	101	101	95
3000	107	116	109	116	112	125	125	119	114
3000	125	125	116	117	122	133	136	131	130
3000	149	141	151	140	148	147	136	137	129
3000	161	162	146	155	148	156	140	146	153
3000	143	143	152	156	163	156	165	157	154

Table C-16. Sample F5 hemocytometer cell counting

Dilution	Trial 1 # of cells count (cells)			Trial 2 # of cells count (cells)			Trial 3 # of cells count (cells)		
	Quadrant 1	Quadrant 2	Quadrant 3	Quadrant 1	Quadrant 2	Quadrant 3	Quadrant 1	Quadrant 2	Quadrant 3
150	65	67	71	68	70	65	66	63	64
150	91	91	87	83	84	84	86	84	88
250	64	67	65	62	61	56	61	59	66
500	161	164	159	173	174	164	166	162	177
3000	81	86	85	85	80	85	88	89	84
3000	124	120	134	115	125	117	134	127	122
3000	131	137	149	140	140	130	152	146	145
3000	152	160	158	161	152	163	147	148	139
3000	159	151	159	165	165	149	143	149	156
3000	155	162	156	142	143	151	164	156	154

Table C-17. Sample F6 hemocytometer cell counting

Dilution	Trial 1 # of cells count (cells)			Trial 2 # of cells count (cells)			Trial 3 # of cells count (cells)		
	Quadrant 1	Quadrant 2	Quadrant 3	Quadrant 1	Quadrant 2	Quadrant 3	Quadrant 1	Quadrant 2	Quadrant 3
150	54	53	55	57	58	58	54	53	51
150	75	76	70	69	66	73	73	75	73
250	52	54	52	50	50	48	52	50	53
500	89	89	93	91	82	86	85	96	88
3000	43	42	40	46	44	48	41	43	42
3000	59	56	61	59	64	63	60	59	55
3000	63	57	61	66	66	66	59	64	61
3000	61	62	68	67	65	60	65	67	66
3000	68	73	67	71	66	66	61	62	67
3000	67	70	67	68	66	71	66	65	63

Table C-18. Sample F7 hemocytometer cell counting

Dilution	Trial 1 # of cells count (cells)			Trial 2 # of cells count (cells)			Trial 3 # of cells count (cells)		
	Quadrant 1	Quadrant 2	Quadrant 3	Quadrant 1	Quadrant 2	Quadrant 3	Quadrant 1	Quadrant 2	Quadrant 3
150	55	55	53	58	59	59	55	55	57
150	74	77	75	70	67	74	76	78	72
250	53	50	54	51	51	49	53	55	53
500	87	98	89	93	83	88	91	91	94
3000	42	44	42	46	45	49	44	43	41
3000	53	52	48	52	56	56	52	49	54
3000	53	57	54	58	59	58	55	51	54
3000	57	58	58	58	57	52	53	54	59
3000	52	53	57	60	56	56	58	62	57
3000	55	54	52	57	55	59	56	59	56

Table C-19. Sample F8 hemocytometer cell counting

Dilution	Trial 1 # of cells count (cells)			Trial 2 # of cells count (cells)			Trial 3 # of cells count (cells)		
	Quadrant 1	Quadrant 2	Quadrant 3	Quadrant 1	Quadrant 2	Quadrant 3	Quadrant 1	Quadrant 2	Quadrant 3
150	52	51	53	56	56	55	56	58	57
150	89	83	79	82	80	89	76	83	79
250	61	57	61	54	57	58	59	60	55
500	38	37	38	36	35	33	34	36	36
3000	26	24	26	23	24	23	25	26	26
3000	28	28	27	27	28	28	26	25	26
3000	29	29	28	30	27	29	26	28	28
3000	29	28	29	29	30	31	31	31	29
3000	28	30	30	29	31	30	34	30	31
3000	30	33	31	30	29	29	32	30	32
3000	29	30	31	32	33	31	33	32	32
3000	33	32	30	30	31	32	32	31	33
3000	29	31	28	34	31	33	31	32	33

Table C-20. Sample F9 hemocytometer cell counting

Dilution	Trial 1 # of cells count (cells)			Trial 2 # of cells count (cells)			Trial 3 # of cells count (cells)		
	Quadrant 1	Quadrant 2	Quadrant 3	Quadrant 1	Quadrant 2	Quadrant 3	Quadrant 1	Quadrant 2	Quadrant 3
150	56	56	54	56	54	55	52	55	55
150	80	73	77	70	74	69	66	69	70
250	53	49	51	49	50	47	49	51	53
500	45	40	44	41	45	42	40	40	40
3000	20	19	19	19	19	20	17	18	17
3000	22	22	21	20	21	22	22	22	22
3000	23	23	22	23	21	23	20	21	21
3000	22	21	21	21	23	22	22	22	22
3000	21	20	21	22	24	22	23	22	23
3000	20	22	21	22	22	21	23	22	23

Table C-21. Sample F10 hemocytometer cell counting

Dilution	Trial 1 # of cells count (cells)			Trial 2 # of cells count (cells)			Trial 3 # of cells count (cells)		
	Quadrant 1	Quadrant 2	Quadrant 3	Quadrant 1	Quadrant 2	Quadrant 3	Quadrant 1	Quadrant 2	Quadrant 3
150	52	55	55	56	54	55	56	56	54
150	66	69	70	70	74	69	80	73	77
250	49	51	53	49	50	47	53	49	51
500	41	41	40	41	46	42	46	41	44
3000	20	21	20	22	22	23	24	23	23
3000	26	25	26	23	24	25	25	25	24
3000	23	24	24	27	24	26	26	26	25
3000	26	25	26	25	26	26	25	24	24
3000	27	25	26	25	28	25	24	23	24
3000	26	25	27	25	25	24	23	25	24

Appendix D: Row data of SSF method

D.1 SSF detailed results

Table D-1. SSF method measurement for BC production triplicates and the final pH *

Sample	Trial 1			Trial 2			Trial 3		
	W_o [a] (g)	W_f [b] (g)	pH	W_o [a] (g)	W_f [b] (g)	pH	W_o [a] (g)	W_f [b] (g)	pH
S1	14.5794	14.6668	3.0	14.8924	14.9749	3.1	14.0789	14.1630	3.3
S2	15.0390	15.1116	3.2	14.8698	14.9435	3.2	14.0897	14.1592	2.9
S3	14.2117	14.2688	2.2	13.7163	13.7729	2.4	15.0742	15.1351	2.3
S4	14.0747	14.1337	3.0	14.1247	14.1832	3.1	15.0397	15.0992	2.8
S5	14.7366	14.8077	2.9	13.7955	13.8658	2.8	15.5702	15.6445	3.0
S6	13.9668	14.0299	3.1	13.7580	13.8249	3.0	15.0160	15.0843	3.0

** Calculation described in Materials and Methods (Measurement of BC production)*

[a] *Initial weight of crucible before the 1 ml BC sample*

[b] *Final weight of crucible after reaching a final weight of dried 1 ml BC sample*

D.2 SHF sugars quantification analysis

Table D-2. Sample S1 HPLC analysis areas in triplicates

Time (hr)		Area of HPLC absorbance (µV.s)						
		Glucose	**Galactose**	**Mannose**	**Xylose**	**Arbinose**	**Fructose**	**H.Furfural**
Trial 1	0	4651154.02	294212.14	302325.69	3111906.02	844733.54	152.28	10190.71
	12	3630496.41	224887.77	228764.95	3209894.85	674438.60	151.53	9668.50
	24	2844196.52	197535.28	228445.35	2712187.39	514667.93	163.09	10301.00
	35	2473245.89	177333.45	199587.42	2454061.94	452216.88	153.12	9689.81
	48	2093695.79	150655.30	162322.09	2090648.19	431792.22	152.16	11192.11
	60	1747759.65	118911.40	138192.50	1768382.19	379869.75	151.04	9912.99
	95	1002367.08	73310.34	77468.10	997951.36	228707.83	137.43	11242.86
	131	669190.70	48236.42	54128.46	673138.73	149040.35	144.05	10653.50
	157	509958.45	37282.22	41462.15	543334.73	124969.58	134.33	10589.77
	170	442746.35	35630.68	39967.95	529707.94	117377.94	164.01	10174.10
Trial 2	0	5062639.76	287929.23	308922.75	3387215.11	816159.40	160.17	9879.92
	12	3624165.17	228063.89	227424.01	3204297.09	637249.05	162.66	10407.22
	24	2886369.06	212092.86	225149.06	2752402.57	527613.56	148.08	10904.18
	35	2405045.30	168107.26	196012.43	2386390.35	452098.71	150.93	10390.23
	48	2052915.29	140438.52	164950.40	2049927.06	423448.93	143.82	11286.83
	60	1739548.22	124118.55	143759.50	1760073.86	389500.10	152.02	10376.21
	95	955720.53	67724.62	78740.03	951510.31	229602.30	141.00	11176.10
	131	641662.39	49128.48	51774.41	645448.01	148137.82	151.55	10177.20
	157	468218.83	38227.39	43277.78	498863.29	124382.44	140.61	11016.37
	170	441021.54	35513.63	43485.32	527644.35	122908.71	160.98	10501.51
Trial 3	0	4912476.72	292668.92	307302.36	3286746.87	905646.06	159.34	9947.36
	12	3297415.87	211415.29	215559.85	2915402.47	669110.12	157.10	10942.89
	24	3027095.87	213279.67	211544.44	2886597.75	491233.07	163.91	10814.02
	35	2428915.51	175380.16	182690.92	2410075.41	431410.36	145.66	9937.96
	48	2112277.01	154017.37	175625.07	2109202.37	456665.53	143.62	10540.87
	60	1644839.57	126796.09	135886.56	1664247.71	388769.62	146.04	10729.40
	95	1002632.75	71376.69	83779.78	998215.87	213395.15	152.60	10600.84
	131	683662.91	46361.28	56482.50	687696.32	161737.01	152.16	10187.90
	157	523968.47	38155.69	44211.39	558261.68	117610.52	145.40	10413.06
	170	411493.80	39244.74	43222.68	492316.95	117572.93	154.82	10342.99

Table D-3. Sample S2 HPLC analysis areas in triplicates

Time (hr)		Area of HPLC absorbance (μV.s)						
		Glucose	**Galactose**	**Mannose**	**Xylose**	**Arbinose**	**Fructose**	**H.Furfural**
Trial 1	0	5485284.40	320852.47	416304.99	3998385.21	976450.02	148.89	42613.99
	12	5248375.04	293425.64	322250.15	3637313.08	721154.44	155.29	39082.24
	24	4180344.09	276214.42	313979.03	3265831.50	741686.75	141.04	38950.67
	35	3968695.23	274278.75	268164.14	3003732.76	613251.68	146.58	39711.81
	48	3453434.28	239713.95	243592.98	2942047.79	546680.98	156.50	42245.89
	60	2564161.48	217518.50	242927.81	2919308.03	558968.42	153.44	40500.68
	95	2000293.86	134622.42	141109.13	1517647.02	319422.08	153.95	42253.70
	131	1192602.23	90864.69	102102.02	1189528.74	223833.75	153.27	41102.53
	157	1036651.07	75200.34	89648.17	988634.82	193586.62	153.47	38501.09
	170	1051167.18	62890.00	72650.90	925738.31	176002.21	148.90	39320.34
Trial 2	0	5805402.92	322744.29	410507.35	3714574.41	909680.98	149.15	42688.43
	12	5204328.93	300144.94	339915.98	3697369.22	760718.15	165.06	41138.49
	24	4384377.17	298995.73	312755.86	3213076.53	746116.92	148.38	40979.04
	35	4230363.59	283058.46	289391.16	2992584.39	636923.84	147.28	39903.37
	48	3403275.35	236691.22	244395.84	3007902.43	546840.66	151.28	40837.02
	60	2652624.53	217536.24	247712.68	2932546.14	514887.21	155.29	40989.75
	95	1980575.51	129081.00	147288.33	1631016.09	305553.52	151.67	41628.86
	131	1177767.98	97229.30	104471.95	1203137.82	237083.24	153.03	41037.72
	157	1033081.48	72088.44	86038.50	955183.97	205009.84	164.97	41385.04
	170	996000.84	61582.64	75403.58	947089.55	174057.34	158.70	41907.26
Trial 3	0	5759536.68	304114.38	385914.86	4204186.38	943065.50	135.47	38771.98
	12	4901503.03	300747.12	348539.93	3411761.70	792216.04	160.72	40851.87
	24	4385539.24	291109.33	291815.90	3474860.65	703510.33	148.98	41142.89
	35	4096539.12	278878.50	292374.36	2791152.16	624322.59	153.02	41457.42
	48	3198269.74	221899.27	269185.21	2740060.66	535855.40	151.85	40991.49
	60	2573860.57	238849.36	227089.89	2760910.39	541037.72	149.96	39582.17
	95	2117587.51	143340.65	150465.70	1600184.49	324793.92	146.43	40191.83
	131	1131604.71	94504.04	104712.68	1203990.13	239478.00	145.18	38932.35
	157	1079906.11	81191.08	90873.17	968327.45	201163.18	164.18	41186.48
	170	1084910.10	58637.16	81042.89	1004697.61	162647.89	150.89	39844.99

Table D-4. Sample S3 HPLC analysis areas in triplicates

Time (hr)		Area of HPLC absorbance (µV.s)						
		Glucose	**Galactose**	**Mannose**	**Xylose**	**Arbinose**	**Fructose**	**H.Furfural**
Trial 1	0	7098331.56	446969.16	457323.66	5059249.80	1027881.76	154.99	9979.10
	12	5505246.72	371315.11	437509.57	4616725.71	729098.75	153.22	10837.43
	24	4440398.40	320518.17	363758.52	4049616.91	697751.86	146.38	10502.30
	35	3963411.93	283788.11	337440.94	3413140.96	528889.43	140.52	10746.07
	48	2987111.81	242385.14	287423.98	3353455.89	560743.39	152.50	9422.48
	60	2758402.91	207237.67	256077.88	2849664.92	509069.76	144.24	10902.17
	95	2110699.77	160331.34	193531.00	2428431.42	376133.98	146.95	9576.70
	131	1948516.95	142089.22	166198.86	2019726.57	366878.92	150.79	10334.95
	157	1865565.87	132545.79	157683.59	1952947.81	322248.83	148.24	10538.59
	170	1728942.92	126909.33	149901.37	1950090.71	320544.97	155.04	10902.17
Trial 2	0	6861607.50	411829.81	444596.60	5219359.07	1016523.40	151.76	10011.99
	12	5398016.87	362891.89	416627.86	4559194.48	772121.65	146.88	10475.25
	24	4500152.77	323030.32	369899.66	4379473.36	725924.39	144.26	10838.48
	35	3813206.86	279882.08	334526.12	3590881.46	567866.53	149.34	10208.89
	48	3108303.64	243854.49	274011.51	3403289.77	533958.49	144.66	9427.29
	60	2874511.20	209321.65	252364.75	3067392.61	483825.26	132.94	10991.55
	95	2221195.61	173647.10	189763.80	2442936.72	395809.92	156.27	9669.53
	131	1941285.02	149429.19	172526.76	1947416.38	366764.91	155.62	10295.24
	157	1768371.04	132556.61	163761.61	1850704.05	324310.15	145.58	10153.86
	170	1731418.68	131007.80	157176.63	1915882.76	290515.16	147.81	10746.07
Trial 3	0	6887561.94	436263.32	448889.74	4800433.13	949890.34	144.51	11027.52
	12	5554104.91	372170.71	436636.57	4162077.82	757634.11	165.43	10706.52
	24	4139191.97	309480.13	366201.34	4242508.12	746400.02	139.19	10678.42
	35	4036289.70	263025.53	310522.08	3605239.32	571044.13	142.83	11064.23
	48	3302845.12	229818.32	273965.45	3268065.62	576100.00	152.75	9167.03
	60	2736479.51	227970.03	243508.27	3225274.59	511006.78	142.04	10125.48
	95	2228913.74	171610.45	206558.90	2303434.17	407763.50	152.01	8770.57
	131	1810616.23	142799.16	167978.22	1941600.17	406439.82	153.32	11389.01
	157	1755795.00	145543.84	157642.07	1948997.66	311615.58	162.77	11326.75
	170	1647801.46	140121.56	157300.46	1861360.87	317696.78	155.09	10370.96

Table D-5. Sample S4 HPLC analysis areas in triplicates

Time (hr)		Area of HPLC absorbance (μV.s)						
		Glucose	**Galactose**	**Mannose**	**Xylose**	**Arbinose**	**Fructose**	**H.Furfural**
Trial 1	0	7315266.53	292637.70	304248.12	3158620.50	842263.56	158.83	10390.23
	12	5352615.98	272434.15	265936.79	3093370.41	642863.69	147.88	10432.03
	24	4858836.01	236792.03	227443.83	2851194.41	583453.21	159.31	10811.54
	35	3748318.47	201184.05	218801.69	2744194.23	531186.52	150.29	10602.78
	48	3380522.92	188281.03	185291.44	2333119.83	495306.93	148.35	9539.39
	60	2961597.14	128982.50	177052.24	2170545.43	441946.52	156.48	9973.51
	95	2313721.32	131049.38	136232.25	1740138.94	393396.97	154.19	9483.67
	131	2038569.79	96992.63	134957.62	1604744.40	353317.21	154.44	10410.26
	157	1928524.07	88038.85	120908.03	1350646.11	340656.99	147.41	9836.56
	170	1958162.06	87085.79	124503.93	1142171.17	253776.13	148.58	10498.76
Trial 2	0	6953177.40	297862.73	312878.41	3359292.77	878913.54	158.64	10071.50
	12	5523526.79	262393.34	267764.60	3067819.59	632620.33	158.18	10147.89
	24	4617630.52	237443.56	225998.19	2840006.33	583561.11	154.74	9912.99
	35	3897423.45	197473.33	229586.02	2452158.32	525950.23	157.37	9979.10
	48	3148491.32	180660.04	194984.23	2321775.11	491003.07	147.85	9617.61
	60	2879652.17	143682.04	173394.70	2143546.98	412757.11	149.50	10530.40
	95	2318561.14	120513.14	147945.22	1697442.57	396480.33	158.59	9372.06
	131	2124096.29	93711.74	139644.70	1558261.68	343574.37	151.47	10011.99
	157	2076372.68	93672.23	127964.16	1393685.38	320618.74	146.12	10705.55
	170	1950533.62	89215.83	113350.59	1212608.90	270486.31	154.22	9668.50
Trial 3	0	7344516.07	284309.86	289417.07	3267954.74	837857.41	151.11	9556.27
	12	5771014.53	265152.20	249648.34	3191918.50	684466.24	156.68	10438.67
	24	4601675.29	225183.81	218540.93	3141495.70	546563.20	153.83	10294.07
	35	3967096.61	189909.84	220413.33	2536284.25	562334.35	150.54	10436.72
	48	3399439.27	187335.36	200872.81	2105158.66	476692.27	163.38	8859.80
	60	2907947.07	142441.51	186755.18	2059529.47	443274.70	148.24	10514.69
	95	2261726.47	135069.32	152884.61	1620151.31	379849.12	148.16	9161.07
	131	2153121.12	103986.63	128839.60	1571432.89	346984.37	140.27	10596.36
	157	2043730.25	91023.90	126953.17	1372236.96	335321.87	141.99	10476.49
	170	1819939.45	94392.13	114676.87	1227528.02	261908.97	150.05	10851.34

Table D-6. Sample S5 HPLC analysis areas in triplicates

Time (hr)		Area of HPLC absorbance (μV.s)						
		Glucose	**Galactose**	**Mannose**	**Xylose**	**Arbinose**	**Fructose**	**H.Furfural**
Trial 1	0	5567048.83	289679.99	357316.06	3663428.46	916391.51	144.64	49817.40
	12	4572446.29	241619.41	368190.12	3095257.26	827673.60	147.66	48470.91
	24	4471689.30	229414.37	267148.48	2849174.43	804712.79	147.20	51379.65
	35	4173762.34	213231.41	235693.85	2626164.93	671678.93	154.72	51379.65
	48	3698077.82	191173.82	209164.90	2374424.44	602197.53	141.98	48431.52
	60	3104912.45	174657.78	183736.53	2393458.84	460261.72	150.99	48040.50
	95	1942267.94	116425.10	119737.70	1437369.46	297864.77	148.27	50764.84
	131	1464345.64	85416.52	87562.24	1072809.72	220155.60	148.28	49251.67
	157	1267452.26	77462.92	70001.53	850017.72	204269.00	150.96	51816.47
	170	1196654.21	67610.71	69531.77	893632.26	190629.92	139.79	54372.59
Trial 2	0	5424914.80	306047.82	382130.34	3579784.72	958764.85	146.08	52833.19
	12	4832282.48	256905.19	343019.29	3258563.07	891014.70	143.02	52125.98
	24	3993990.94	220720.04	263268.31	2947843.17	774215.85	154.93	48811.28
	35	4173359.55	202387.13	231173.52	2433672.96	637519.45	148.50	52329.84
	48	3505442.75	182571.19	213095.31	2300397.01	575099.25	142.45	44514.07
	60	3300935.47	174652.67	187702.31	2181726.24	489027.48	156.65	47799.16
	95	2114521.53	106977.87	121483.55	1499957.92	319395.68	138.21	48740.96
	131	1372212.46	79427.11	84790.07	994830.38	224034.33	157.98	53510.69
	157	1231501.36	78860.60	66001.34	870975.30	207954.66	150.44	49224.12
	170	1165575.94	66312.41	71480.93	810750.16	185674.75	149.83	50214.11
Trial 3	0	5225260.87	296235.62	371219.60	3423182.82	946535.64	140.25	54443.61
	12	4787481.45	260501.97	351427.79	3087641.15	913815.26	131.76	52494.92
	24	4128972.02	233632.75	247409.63	3187569.93	819509.56	151.12	52900.87
	35	3983071.79	213472.98	237715.13	2573139.15	672439.88	156.82	49382.31
	48	3511404.53	182874.47	201153.61	2338584.07	576054.60	156.90	48139.01
	60	3251092.78	161638.79	200824.31	2339661.34	481368.69	169.95	52249.13
	95	2171795.44	106151.78	127880.08	1522647.00	305492.87	148.14	48583.00
	131	1527909.01	86021.08	86282.79	1057533.87	202397.80	138.58	50329.44
	157	1162792.12	75243.91	70520.97	912486.13	198417.50	157.26	52051.21
	170	1284956.85	61408.29	75116.89	817129.58	170623.29	150.34	48505.10

Table D-7. Sample S6 HPLC analysis areas in triplicates

Time (hr)		Area of HPLC absorbance (μV.s)						
		Glucose	**Galactose**	**Mannose**	**Xylose**	**Arbinose**	**Fructose**	**H.Furfural**
Trial 1	0	4995097.26	289310.05	333065.20	3552645.64	849468.13	156.45	63274.64
	12	4351730.59	298404.38	352250.40	3527548.08	755816.22	157.47	59938.21
	24	4017509.28	271105.21	242761.76	3096895.29	702300.15	162.17	60060.01
	35	4003489.21	210638.60	228583.36	2546741.53	544397.63	158.72	59403.62
	48	3617514.57	191402.89	203842.60	2463098.09	520726.65	152.08	56637.96
	60	3002010.83	158836.55	186152.46	2129378.39	470334.73	156.04	61118.28
	95	2121067.13	112225.77	128135.11	1527461.47	299608.94	149.77	60983.99
	131	1523925.46	80630.98	88848.42	988588.29	223467.56	159.50	61324.68
	157	1214886.09	64279.69	69252.53	906571.71	179983.13	147.78	58457.09
	170	1219363.49	64516.59	72690.19	832710.01	184204.58	146.40	61654.00
Trial 2	0	5253311.40	304265.51	342637.46	3471458.21	832466.68	162.39	63039.80
	12	4136878.14	283671.64	350011.49	3533119.42	770708.20	146.85	62516.51
	24	4051765.62	273416.86	259180.77	3251294.82	718912.79	166.42	58908.86
	35	3650715.54	192077.85	238173.38	2671400.90	579078.87	155.32	61387.00
	48	3362295.85	177899.25	211699.85	2455365.05	493663.88	151.62	58419.03
	60	3147335.82	166525.71	176224.42	2240852.06	470094.90	152.51	62546.34
	95	2064851.01	109251.38	126410.60	1527743.94	314180.38	162.74	61622.82
	131	1486100.08	78629.63	88090.91	1089302.93	208090.76	154.78	58493.07
	157	1255448.97	66425.87	73664.31	887199.08	188793.04	152.91	60909.61
	170	1185739.13	62737.52	68908.31	892901.77	180905.12	144.30	61637.89
Trial 3	0	5225870.34	302676.15	368923.74	3472190.15	907117.69	174.51	58796.56
	12	4520442.02	309973.17	338462.16	3346573.10	815104.71	150.63	59654.48
	24	3727799.10	251555.30	253070.20	3342260.67	702260.37	153.60	64140.92
	35	4058068.40	213510.21	241427.92	2546313.45	601958.13	150.29	60317.98
	48	3471406.55	183672.30	203788.92	2565124.33	533937.91	144.27	63049.81
	60	3094246.17	163716.73	185391.59	2248638.49	428991.54	146.01	58444.58
	95	2079138.64	110007.34	116716.91	1430884.62	314367.25	157.23	56500.59
	131	1419280.45	75094.20	85538.06	1093710.61	224635.15	166.82	60290.25
	157	1233585.96	65269.10	76574.77	858419.57	179952.87	151.03	61741.91
	170	1197507.65	63360.19	71889.52	854035.09	168610.34	139.27	58817.30

D.3 SSF cells counting

Table D-8. Sample S1 hemocytometer cell counting

Dilution	Trial 1 # of cells count (cells)			Trial 2 # of cells count (cells)			Trial 3 # of cells count (cells)		
	Quadrant 1	Quadrant 2	Quadrant 3	Quadrant 1	Quadrant 2	Quadrant 3	Quadrant 1	Quadrant 2	Quadrant 3
150	53	58	57	56	54	56	55	53	52
150	77	73	73	79	75	79	70	76	73
250	78	81	81	81	81	84	91	86	84
500	127	125	122	122	113	124	123	134	127
3000	54	52	56	59	59	57	60	64	60
3000	84	85	78	78	84	82	89	82	91
3000	119	119	113	116	114	123	111	113	110
3000	128	121	133	124	131	129	124	124	115
3000	126	133	129	138	131	131	122	121	126
3000	127	133	132	131	135	126	125	115	125

Table D-9. Sample S2 hemocytometer cell counting

Dilution	Trial 1 # of cells count (cells)			Trial 2 # of cells count (cells)			Trial 3 # of cells count (cells)		
	Quadrant 1	Quadrant 2	Quadrant 3	Quadrant 1	Quadrant 2	Quadrant 3	Quadrant 1	Quadrant 2	Quadrant 3
150	57	54	53	57	56	57	55	59	59
150	103	113	108	117	112	117	113	109	108
250	78	74	72	69	70	72	67	70	69
500	93	101	96	93	86	94	96	95	92
3000	55	58	55	54	54	52	50	47	51
3000	71	65	73	62	67	65	67	68	62
3000	84	86	83	87	86	93	90	90	86
3000	94	94	87	94	99	98	97	92	101
3000	92	91	95	104	99	99	95	100	97
3000	95	88	95	100	103	96	96	101	100

Table D-10. Sample S3 hemocytometer cell counting

Dilution	Trial 1 # of cells count (cells)			Trial 2 # of cells count (cells)			Trial 3 # of cells count (cells)		
	Quadrant 1	Quadrant 2	Quadrant 3	Quadrant 1	Quadrant 2	Quadrant 3	Quadrant 1	Quadrant 2	Quadrant 3
150	60	59	60	58	60	60	55	54	59
500	100	101	102	102	107	100	103	97	110
3000	46	47	46	48	48	47	50	49	48
3000	75	78	72	82	78	82	76	77	76
3000	111	110	110	103	104	104	104	104	110
3000	117	129	126	126	118	119	129	125	117
3000	138	147	146	153	146	146	148	146	146
3000	164	148	161	163	163	154	139	155	148
3000	147	154	145	151	147	146	164	161	161
3000	151	154	161	149	143	140	159	162	151

Table D-11. Sample S4 hemocytometer cell counting

Dilution	Trial 1 # of cells count (cells)			Trial 2 # of cells count (cells)			Trial 3 # of cells count (cells)		
	Quadrant 1	Quadrant 2	Quadrant 3	Quadrant 1	Quadrant 2	Quadrant 3	Quadrant 1	Quadrant 2	Quadrant 3
150	63	63	67	67	69	69	69	68	69
150	88	83	93	87	91	85	85	86	87
250	65	64	62	63	62	61	60	61	60
500	164	164	162	175	167	176	161	168	155
3000	83	83	88	82	83	83	89	88	88
3000	130	126	118	126	118	119	117	129	126
3000	143	141	141	148	141	141	133	142	141
3000	138	153	146	161	161	153	162	147	159
3000	165	162	162	152	148	147	148	155	146
3000	160	163	152	149	143	141	152	155	162

Table D-12. Sample S5 hemocytometer cell counting

Dilution	Trial 1 # of cells count (cells)			Trial 2 # of cells count (cells)			Trial 3 # of cells count (cells)		
	Quadrant 1	Quadrant 2	Quadrant 3	Quadrant 1	Quadrant 2	Quadrant 3	Quadrant 1	Quadrant 2	Quadrant 3
150	46	46	46	45	47	46	48	45	46
150	68	72	72	71	69	71	69	67	64
250	46	48	50	51	48	50	51	51	49
500	53	53	55	58	57	54	56	57	57
3000	42	42	39	41	41	41	40	40	43
3000	58	54	59	57	57	57	56	60	55
3000	75	73	80	76	77	72	84	83	81
3000	87	93	89	92	93	94	86	79	86
3000	92	91	90	91	95	94	91	88	82
3000	90	85	84	92	92	88	92	97	99

Table D-13. Sample S6 hemocytometer cell counting

Dilution	Trial 1 # of cells count (cells)			Trial 2 # of cells count (cells)			Trial 3 # of cells count (cells)		
	Quadrant 1	Quadrant 2	Quadrant 3	Quadrant 1	Quadrant 2	Quadrant 3	Quadrant 1	Quadrant 2	Quadrant 3
150	53	56	55	55	55	54	57	54	54
150	74	72	74	71	75	75	72	69	67
250	53	50	52	48	50	51	52	53	51
500	51	50	47	46	46	48	49	50	50
3000	38	37	37	39	39	36	37	37	40
3000	51	52	52	53	49	53	51	54	50
3000	70	71	66	69	68	74	77	77	75
3000	85	86	87	81	86	83	80	74	80
3000	86	90	89	87	86	86	86	84	78
3000	89	89	85	86	82	81	88	93	95