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Pretreatment of fibrous biomass and growth of biosurfactant-producing Bacillus subtilis on biomass-derived fermentable sugars.

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Abstract
Pretreatment of six fibrous biomasses (switchgrass, alfalfa, soy hulls, soy fiber, DDGS and Baggase) and subsequent hydrolysis using cellulolytic enzymes at a 2.5% (v/v) and 5% (v/v) loading 2.5 (v/v) and 5% (v/v) loading was compared for higher amounts of sugars released. Soaking of biomasses of switchgrass, alfalfa, soy hulls and bagasse in 15% w/w ammonia was optimal at 60 °C for 12 h, followed by enzymatic hydrolysis, yielding 72, 70, 80 and 75% carbohydrate conversions, respectively. However, soaking in ammonia was not needed for soy fiber and DDGS as these contained very little lignin. Ultrasonication for 3 min @ 100% amplitude (170 μM) was found to be optimal for soy fiber and DDGS from which 77 and 83% carbohydrate conversion, respectively, was obtained following enzyme treatment at 5% (w/v) enzyme. The sugars released by enzymatic hydrolysis of pretreated biomass were utilized as an energy source by Bacillus subtilis in fermentation media at 2% (w/v) of concentration. In shake flask trials, cell growth was 15-20% higher on hydrolysates of ammonia-treated switchgrass and alfalfa vs. glucose-based control media due to the presence of a wider range of monomeric sugars (glucose, xylose, arabinose, mannose and galactose). In contrast, growth was less on soy hull hydrolysates prepared with ammonia pretreatment.

Keywords
Bacillus subtilis, Biomass pretreatment, Biosurfactants, Enzymatic hydrolysis, Liquid ammonia, Ultrasonication

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Pretreatment of fibrous biomass and growth of biosurfactant-producing *Bacillus subtilis* on biomass-derived fermentable sugars

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Abstract

Pretreatment of six fibrous biomasses and subsequent hydrolysis using cellulolytic enzymes at a 2.5 % (v/v) and 5% (v/v) loading 2.5 (v/v) and 5% (v/v) loading was compared for higher amounts of sugars released. Soaking of biomasses of switchgrass, alfalfa, soy hulls and bagasse in 15% w/w ammonia was optimal at 60°C for 12 h, followed by enzymatic hydrolysis, yielding 72%, 70%, 80% and 75% carbohydrate conversions respectively. However, soaking in ammonia was not needed for soy fiber and DDGS as these contained very little lignin. . Ultrasonication for 3 min @ 100% amplitude (170 uM) was found to be optimal for soy fiber and DDGS from which 77- and 83% carbohydrate conversion, respectively, was obtained following enzyme treatment at 5% (w/v) enzyme. The sugars released by enzymatic hydrolysis of pretreated biomass were utilized as an energy source by Bacillus *subtilis* in fermentation media 2% (w/v) of concentration. In shake flask trials, cell growth was 15-20% higher on hydrolysates of ammonia-treated switchgrass and alfalfa vs. glucose-based control media due to presence of a wider range of monomeric sugars (glucose, xylose, arabinose, mannose and galactose). In contrast, growth was less on soy hull hydrolysates prepared with ammonia pretreatment.

Keywords: Biomass Pretreatment, biosurfactants, liquid ammonia, ultrasonication, enzymatic hydrolysis, Bacillus *subtilis.*

1. Introduction

High lignocellulosic biomass consisting of a complex network of carbohydrates and lignin presents a potential for monomeric sugar extraction and utilization for generation of bio-products. However, crystalline structures of carbohydrates and lignin in fibrous biomass act as barrier to the monomeric sugar extraction as a result of limited enzymatic activity [1]. These challenges have largely been overcome through chemical and physical pretreatments of fibrous biomass both used in isolation and combination. Pretreatment of fibrous biomass has been shown to a) lower the lignin content b) de-crystallize cellulosic structure, and c) increase the surface area to enhance enzymatic release of fermentable sugars. Pretreatment of biomass leads to greater sugar availability that can be utilized to produce valuable fuel and industrial bio-chemicals through fermentation [2].

The sugars generated by pretreatment and enzymatic hydrolysis of fibrous biomass are primarily intended for bioethanol generation through fermentation [3]. However, recent studies have shown that feedstocks such as frying oil wastes, glycerol from bio-diesel production and fermentable carbohydrates from agricultural wastes can be used to produce value-added biochemicals such as biosurfactants. Surfactants are ampiphilic compounds that contain a hydrophilic head and a hydrophobic tail. Those produced by microorganisms or enzymes are known as biosurfactants, and consist of fatty acid chains attached to sugar moieties or a chain of amino acids that define their structure and function. Biosurfactants display high dispersion- and surface tension-

lowering ability compared to synthetically-produced, petroleum based industrial surfactants, and are biodegradable, thereby making them more environmentally attractive [4].

Surfactin is a biosurfactant commonly produced as a secondary metabolite by the bacterium *Bacillus subtilis* subspecies subtilis. It is a cyclic lipopeptide consisting of a fatty acid chain of 12-14 carbons linked to a cyclic heptapeptide. It is one of the most powerful biosurfactant and can lower the surface tension of water from 72 mN/m to 27 mN/m at a low concentration of 20μM [5]. Although it has excellent surface tension lowering capacity, it has low water solubility due to the presence of hydrophobic amino acids in the cyclic amino acid chain [5]. Modular Genetics, Inc. (Woburn, MA) has produced a surfactin variant with higher water solubility using a genetically-modified strain derived from the surfactin-producing *B. subtilis*. The variant has been termed Fatty Acyl-Glutamate (FA-Glu) [6] and differs in having a single glutamic acid residue instead of surfactin's heptapeptide.

Studies have shown that biosurfactants such as fengycins, surfactin and surfactin variants can be successfully produced by bacteria grown on a variety of feedstocks containing 2-7% (w/v) carbon from agricultural by- products and feedstocks. Thavasi et al (2011) utilized 2% (w/v) peanut oil concentration to produce 5.35 g/L of a surfactin-like biosurfactant. De Faria et al (2011) reported producing 230 mg/L of surfactin (C_{14}/Leu_7) by utilizing 5% w/v of biodiesel-derived glycerol as the sole carbon source [5, 7, 8] Such studies have shown the techno-economic and environmental benefits of cellulosic carbon sources over glucose [6]. Although, a wide range of titers on different carbon sources has been reported [4, 5, 8, 9], the abundance and of fibrous feedstocks

justifies effortsto optimize biosurfactant production by fermentation of non-conventional feedstocks such as composite hydrolysates derived from these sources. The choice of feedstock pretreatment and hydrolysis protocols is key in obtaining high monosaccharide and minimizing inhibitory compounds in hydrolysates.

Liquid ammonia pretreatment is one of the most effective delignification techniques and has been used to pretreat fibrous and ligncellulosic biomass such as corn stover [10]. Ultrasonication is a physical pretreatment method that has been relatively unexplored but has potential to alter biomass structure by de-crystallizing the cellulosic matrix with maximum retention of biomass and polysaccharides initially present [11]. Both pretreatment techniques were utilized singly and in combination on six fibrous feedstocks to determine the optimum pretreatment conditions. These were then used to generate soluble sugars that were substituted for glucose in bacterial growth media.

In this study, biomass pretreatment conditions (i.e. Ammonia concentration and solid loading) were chosen from the work of Kim et al (2003).Optimization of the reaction time, pretreatment temperature and enzymatic loading was performed for hydrolysate preparation. Hydrolysates derived from these materials were then tested as carbon sources for the growth of biosurfactant-producing strains of *B. subtilis*. Using a variety of monosaccharides in biomass hydrolysates as an energy source presents a potential to assess growth of surfactant-producing bacteria for sustainability of the process [12].

Materials and methods

2.1 Materials

Six fibrous feedstocks were utilized in this study, namely switchgrass (SW), alfalfa (AA), bagasse (BG), soy hulls (SH), soy fiber (SF) and distillers' dry grains with soluble (DDGS). Switchgrass and alfalfa were harvested in June 2010 and August 2010 respectively from the Bio Century Research Farm, Iowa State University. Soy hulls were obtained from Processing, Inc., Eagle Grove, IA. Soy fiber was obtained from an integrated countercurrent 2-stage Enzyme-Assisted Aqueous Extraction Process (EAEP) after separating the soy skim, and frozen at -20°C [13]. DDGS was obtained from Lincolnway Energy (Nevada, IA). Bagasse from the 2012 sugarcane harvest was obtained from the Agricultural Center at Louisiana State University. All feedstocks were dried in a convection step oven at 105°C for 12 h, ground to 2mm particle size in a Wiley ball mill and stored in Ziploc bags at 25°C. The enzymes cellulase (NS22086), hemicellulase (NS22083) and a mix of pectinase/arabinase/xylanase enzymes (NS22119), were obtained from Novozymes, Inc., Franklinton, NC. The activities for these three enzymes were 1000 BHU (2)/g, 2500 FXU-S/g, 13700 PGU/g (Novozyme © NS22086, NS22083, and NS22119, respectively). The surfactant producing strains *Bacillus subtilis* T1651 and the *Bacillus subtilis* E4088 were obtained from Modular Genetics Inc., Woburn, MA.

2.2 Selection of best conditions for pretreatment and enzyme loading for highest carbohydrate conversion

Initially both liquid ammonia preatreatment and ultrasonication were used for each feedstock separately Liquid ammonia pretreatment consisted of soaking each of the six feedstocks in aqueous ammonium hydroxide solution, wherein five gram of ground, dried biomass were mixed with 55 mL of 15% (v/v) ammonium hydroxide solution in 250-mL

screw cap Erlenmeyer flasks. They were heated at 40, 60 and 80°C in a water bath for 12 h, and at 121°C in an autoclave for 1 h. Samples were cooled to room temperature, then vacuum filtered to remove all liquid. During filtration the slurries were washed with cold deionized distilled water (DDW) to remove the ammonia as verified by monitoring the filtrate pH till it was that of wash water. The filtered solid biomass was then weighed and packed into Ziploc bags and stored at 4°C as per National Renewable energy laboratory's laboratory analytical protocol recommendations for enzymatic hydrolysis of pretreated samples [14, 15]. All reactions were performed in triplicates.

The ultrasonication pretreatment was then performed on all six feedstocks in a BRANSON© 2000 EA ultrasonicator with a 1:1.25 booster. Five gram of fresh dried feedstock were mixed with 50 mL of deionized distilled water in 250-mL beaker kept in an ice bath to prevent heat buildup due to the sonication process [11]. Ultrasonication was performed for 1 and 3 min respectively and the pretreated slurries were filtered, stored and weighed in the same manner as described above. Moisture content was determined in a convection oven at 105°C for 12 h. Two g equivalent dry solids of pretreated samples were suspended in 50-mL of 0.1 M Na-acetate buffer, pH 5.0 in a 250-mL screw-capped Erlenmeyer flask, then hydrolyzed in a 1:1:1 mixture of enzymes at loadings of 2.5% and 5% (w/v) for 24 h in shaker-incubator at 50°C, 150 rpm.

The hydrolysate supernatants and the residual solids were weighed and stored in Ziploc bags at -20°C. Carbohydrate conversion was defined as the conversion of biomass solids to soluble feedstocks (CHO), based on the starting dry weight CHO content. It has been defined as the ratio of milligrams of total carbohydrate extracted from one-gram dry

pretreated biomass to the milligrams of total carbohydrate in one- gram dry un-pretreated biomass.

2.3 Preparation of hydrolysate media

Best pretreatment combinations of liquid ammonia treatment and ultrasonication specific to each feedstock were selected based on maximum carbohydrate conversions. These were scaled up to generate larger volumes of hydrolysate to meet growth media requirements for the two *Bacillus subtilis* strains. Thirty g dried, ground feedstock was first treated with 330 mL of 15% ammonium hydroxide (v/v) with ammonia. This pretreated slurry was then ultrasonicated for 3 minutes. Ten g of dry equivalent of the wet recovered biomass after ultrosonication, by calculating the moisture content in these samples, were then hydrolyzed at the optimum enzyme loading of 5% (w/v). The hydrolysates were analyzed for total feedstocks and substituted for glucose equivalent of 2% feedstocks in the growth media.

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2.4 Growth and fermentation of *Bacillus subtilis* strains on hydrolysates and glucose based media.

A glucose-based control growth media, termed S-7 media, was utilized for *Bacillus subtilis* growth. The complete S-7 medium contained (per liter) 2.18 g KH_2PO_4, 14.63 g K_2HPO_4, 1.32 g $(NH4)_2SO_4$, 2.94 g glutamic acid, 20 g glucose, 0.73 mg HCl, 0.49 g $MgCl_2$-$6H_2O$, 14.7 mg $CaCl_2.2H_2O$, 9.9 mg $MnCl_2$-$4H_2O$, 0.14 mg $ZnCl_2$, 1.35 mg $FeCl_3$-$6H_2O$ and 0.67 mg thiamine-HCl. A solution containing phosphate buffer salts (pH 7.5) and ammonium sulfate was prepared and separately sterilized by autoclaving. [7

Prior to inoculation in S-7 media inocula were prepared by growing the cells overnight in seed media at containing 0.6% Na2HPO4, 0.3% KH2PO4, 0.05% NaCl, 0.1% NH4Cl, 0.3% yeast extract at 30° C and 170 rpm in an Innova 4300 shaker-incubator to an initial A650 of 0.01-0.1. A total volume of 50 mL fermentation media was used in all experiments including glucose- and hydrolysate based media, all at 2% (w/v) sugar level, with 0.2 mL inocula volume. Hydrolysate-based media were prepared by measuring the volume that would contain sugars equivalent to 2% (w/v) in the final media. The enzyme hydrolysates were heated for 100°C for 15 min to deactivate the enzymes present prior to media preparation. Fermentations were carried for 72 h at 37° C and 170 rpm in an Innova 4300 shaker-incubator (Eppendorf, New Jersey, NJ). As growth absorbance of a bacterial culture is a good indicator cell density and growth, periodic sampling of fermentation cultures was done and growth determined by measuring the absorbance at 650 nm [16].

2.5 Analytical tests

Untreated raw fibrous feedstock biomass was analyzed total lignin content (%w/w)% moisture of oven-dried biomass (%w/w) according to the NREL LAP procedure. The moisture content was measured by heating samples for 24 h at 105°C in convection oven [14]. Total carbohydrate content in the hydrolysates was analyzed by the Phenol-Sulphuric acid assay from Dubois et al (1956) [17]. This assay involved adding, 0.5 mL 5% phenol solution to 0.5 mL of sample, followed by 2.5 mL 18M H_2SO_4. The resulting mixture was cooled and vortexed before measuring the absorbance at 490 nm. Sugars in the hydrolysates were measured by HPLC conducted isocractically with 0.005 M

sulphuric acid as the mobile phase on an Accela 60057 HPLC unit equipped with a HyperREZXP carbohydrate H+ 8 µm column (Fischer Scientific)

2.6 Statistical Analysis

SAS © 9.4 version was used to conduct the Tukey's least square means analysis for pairwise comparisons for all small scale pretreatment experiments to determine the statistically significant higher values of carbohydrate conversion at a significance level of $p<0.05$.

3. Results and Discussion

3.1 Compositional analyses of untreated fibrous biomasses

Compositional analyses of untreated fibrous feedstock (Table 1), shows the moisture content, acid soluble- and acid insoluble lignin and total feedstocks content of 6 untreated feedstocks. These had average moisture content of 5.7% after 48 h of heating. The highest % total acid soluble- and % total acid insoluble lignin were found to be in switchgrass at 3.47 ± 0.2 and 22.3±1.3% and, respectively.

Feedstock selection was based on a range of lignin- and carbohydrate content for optimizing pretreatment conditions. Switchgrass, alfalfa and bagasse had the highest lignocellulosic contents which that reflected the maximum carbohydrate yields that could be achieved post pretreatment and enzymatic hydrolysis. Sugars were more easily extracted from feedstocks with less, e.g., soy hulls and soy fiber; carbohydrate was less easily extracted from DDGS as its overall total carbohydrate content was the lowest compared to other feedstocks.

3.2 Optimization of pretreatment scheme

Soy fiber and DDGS received no ammonia pretreatment as most of the solid biomass from this material dissolved in 15% liquid ammonia and solid recoveries of <20% were obtained during preliminary experiments. Pretreatment optimization for the other four feedstock resulted in maximum carbohydrate conversions at 60°C and 5% (w/v) enzyme loading. Feedstock carbohydrate conversions from the four ammonia-pretreated feedstocks were significantly ($p<0.05$) better at (5% w/v) enzyme loading compared to 2.5% enzyme loading (Table 2). For switchgrass, alfalfa, soy hulls and bagasse, the optimum treatment combination was at ammonia at 600C and 5% (w/v) enzyme loading For soy fiber, optimum pretreatment was ultrasonication for 3 min at 100% amplitude (170 μm) at 5% (w/v) enzyme loading With DDGS, sonication for 3 min at 5% (w/v) enzyme loading was shown to be optimal. Although there was no significant difference between 1- and 3 min sonication, the longer time produced lower standard deviations among replicates. These results could be attributed to lignin reduction and opening up of the lignocellulosic matrix for enhanced enzymatic activity. Ultrasonication pretreatment, not involving any liquid ammonia treatment for switchgrass, alfalfa, soy hulls and bagasse generated very low CHO yields similar to those of the un-pretreated enzyme hydrolyzed controls at 1- and 3 min, indicating that there was no significant pretreatment effect of sonication on lignocellulosic biomass. Some of the pretreatment combinations tested (e.g. for soy fiber, soy hulls and DDGS) have not been conducted prior to this study. However, liquid ammonia pretreatment of corn stover was done by Kim et al (2003) who observed 85-96% (w/w) carbohydrate conversions in 72 h hydrolysates (Kim

et al., 2003), compared with our results of 65-80% (w/v) carbohydrate conversions yields after 24 h. As such, their data are in accordance with ours [10].

Pretreatment conditions optimized for switchgrass, alfalfa, soy hull and bagasse were combined with 3 min sonication at 100% amplitude to achieve the best results. For DDGS and soy fiber only sonication was used, as ammonification of this material results in intolerable loss of solids. When treatment combinations were scaled up to 30g samples, the feedstock carbohydrate conversions were :a) switchgrass- 59.38 ± 3.6% b) alfalfa- 60.38 ± 1.8 % c) soy hulls- 84.5% ± 3.9 d) soy fiber - 80.08 ± 3.8% e) DDGS- 60.37±10.8, and f) Bagasse 60.2 %. These values are consistent with data from smaller scale trials where higher carbohydrate conversions were achieved with feedstocks containing lower lignin content. The lower conversions for switchgrass, alfalfa, and bagasse could be attributed significantly higher lignin content compared to other three biomasses and to greater losses during total solid recovery during washing and filtration compared to the lower 5g scale experiments. As more washing steps were involved in getting the pH close to neutral for 30 g samples, higher losses were incurred during particle scraping and recovery.

3.3 Distribution and utilization of hydrolysate sugars

Table 3 shows the sugar composition of hydrolysates of pretreated and unpretreated feedstocks. Glucose is the most abundant sugar in the pretreated hydrolysates; other sugars such (e.g., xylose, arabinose, galactose and mannose) were released by enzymatic hydrolysis following pretreatment.. Switchgrass, alfalfa and bagasse had similar monosaccharide compositions consistent with date from Xu et al (2010), Srikanth et al

(1999) and Da Silva et al (2013) who observed high post- pretreatment yields of glucose [18, 19, 20].Pre-treated soy hull hydrolysates had nearly identical percentages of glucose, xylose and mixed sugars, which agree with data, obtained from extruded soy hulls by Karuppuchamy and Muthukumarappan (2013) [21]. . HPLC analyses of sugars from pretreated DDGS and soy fiber also showed similar trend towards maximum glucan conversion as previously observed. The presence of multiple monomeric sugars dominated mostly by glucose in all hydrolysates provided for simultaneous uptake by the bacterial strains or possible competitive uptake that could lead to differences in growth patterns.

3.4 Bacterial growth on biomass hydrolysates

Fifty-mL shake flask fermentations biomass hydrolysates included controls that were enzymatically hydrolyzed without any pretreatment.. They contained insufficient soluble sugars to meet the 2% (w/v) carbohydrate requirement of the media. Media containing 2% (w/v) glucose were also included for comparison except for un-pretreated soy hulls and soy fiber. Maximum absorbance in all shake flasks was 29 h. The absorbance data (Fig 1) suggest that availability of glucose was essential to the growth of the surfactin-producing Bacillus strain; the CSH60 treatment combination for soy hulls consistedcontained more xylose (on a percentage distribution basis), compared to very little xylose present in the enzyme treated hydrolysate of un-pretreated soy hulls followed by alfalfa. Growth in both hydrolysates (Fig1) exceeded that of the glucose control at same carbon levels. Switchgrass and alfalfa hydrolysates showed a similar trend where a preponderance of glucose and availability to other hexose sugars in hydrolysates and lower pentose concentrations resulted in better cell growth as indicated by 15-20% higher

absorbance values than the glucose control. In the ammonia pretreated hydrolysates, maximum growth for both *B. subtilis* strains was observed for switchgrass. .

Growth in soy hull hydrolysates after ammonia pretratement and enzyme. The lower growth on pretreated soy hull hydrolysates could be due to the presence of possible inhibitors formed during alkaline pretreatment. In an earlier study by Reznik et al (2008) unhydrolyzed, ground unpretreated soy hulls were utilized as a carbon source and better growth of an FA-Glu producing strain of B. subtilis on glucose containing media was observed [6]. . Alternatively, an equal distribution of pentoses and hexoses resulting in competitive metabolic uptake could be responsible (Van Foseen et al., 2009) [22]. (Table 2) Since growth on unpretreated and enzyme-treated soy hull and soy fiber (Fig 2) was higher than on 2% (w/v) glucose, the lower growth absorbance on soy hull preatreated hydrolysates could be attributed to the severity of the pretreatment on decreased conversion of glucose. As observed with switchgrass and alfalfa, growth on pretreated DDGS and bagasse hydrolysates was significantly higher than the glucose control; this could also be attributed to the higher percentage of glucose in the media.

4. Conclusion

Pretreatment conditions for six unutilized and under-utilized feedstocks were used to facilitate their use as fermentation feedstocks. Pretreatment optimization of these feedstocks was conducted and hydrolysates of the latter containing monosaccharaides mixtures were tested for growth of biosurfactant producing bacteria. In shake flask trials cell growth was 15-20% higher on pretreated switchgrass and alfalfa hydrolysates vs. 2%

(w/v) glucose and other feedstocks. The significance of this study is that both biosurfactant producing strains were shown to grow on different compositions of monomeric sugars from hydrolysates. It was shown that hydrolysates of alfalfa, switchgrass and bagasse are better at promoting growth of *B. subtilis* strains than other cellulosic biomasses. This provides a good platform to evaluate the growth patterns of bacteria for producing value-added chemicals and to gain a better understanding of optimization of the required feedstock pretreatment. Evaluation of product titers will provide a clearer assessment of the choice of feedstock to achieve higher yields and economic sustainability.

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List of tables and figures.

e) UDD3-Ultrasonicated DDGS for 3 mins and enzyme loading at 5% (w/v)

f) CSBG60- Combined pretreated bagasse at 60C and ultraosonicated for 3 min and enzyme loading of 5% (w/v)

g) All UT combinations are hydrolysates of un-pretreated feedstocks at 5% (w/v) enzyme loading.

4. Figure 1. Comparison of growth (Absorbance at 650 nm) of B. subtillis T5161 (a) and B. subtillis E4088 (b) on glucose vs. combined pretreated of SW, AA, SH and BG. In all media, carbohydrate concentrations were 2% (w/v) (SW-Switchgrass, AA-alfalfa, SH-Soy hulls, BG- Bagasse)

5. Figure 2. Comparison of growth (Absorbance 650 nm) of B. subtillis T5161 (a) and B. subtillis E4088 (b) on glucose vs. ultraosonicated hydrolysates of SF and DDGS. In all media, carbohydrate concentrations were 2% (w/v) (SF- Soy Fiber, DDGS- Dry Distiller's grain solubles).

6. Figure 3. Growth profiles of (Absorbance 650 nm) of B. subtillis T5161 and B. subtillis E4088 (b) on un-pretreated, enzyme hydrolyzed hydrolysates from Soy hulls and Soy Fiber. All other feedsctocks did not generate sufficient carbohydrates without pretreatment.

S-UPSH – Unpretreated hydrolyzate of Soy Hulls for growth of B. subtillis T5161

S-UPSF- Unpretreated hydrolyzate of Soy FIber for growth of B. subtillis T5161

F-UPSH- Unpretreated hydrolyzate of Soy Hulls for growth of B. subtillis E4088

F-UPSF- Unpretreated hydrolyzate of Soy fiber for growth of B. subtillis E40

Table 1

Component	SW	AA	SH	SF	DDGS	Bagasse
Moisture	7.8±0.4	6.3±1.1	3.4±0.22	7.7%±0.8	2.3±1.1	6.8±1.7
Acid soluble lignin	3.47 ± 0.2	2.45 ± 1.2	0.41 ± 0.1	0.003 ± 0.0	0.77 ± 0.0	1.39 ± 0.5
Acid insoluble lignin	22.31 ± 1.3	19.34 ± 1.1	4.22 ± 1.7	1.1 ± 0.0	5.84 ± 1.0	19.45 ± 1.4
Total carbohydrate	80.61 ± 2.7	80.15 ± 1.5	73.38 ± 2.0	77.15 ± 3.7	43.74 ± 2.0	83.17 ± 3.5

Table 2

Treatment	Biomass	Enzyme loading (%)	Solid Recovery	% Conversion
No pretreatment, enzymatic hydrolysis only	SW	2.5	N/A	15.8±5.7^{op}
	AA		N/A	18.5 ± 2.3^{op}
	SH		N/A	31.0± 4.3^{hijklmnop}
	BG		N/A	22.3 ± 3.9^{mnop}
	SF		N/A	44.8 ± 3.5^{efghij}
	DD		N/A	32.0 ± 9.0^{hijklmnop}
	SW	5	N/A	40.8 ± 3.2^{op}
	AA		N/A	24.2 ± 2.4^{op}
	SH		N/A	24.2 ± 3.6^{hijklmnop}
	BG		N/A	22.9 ± 2.3^{mnop}
	SF		N/A	51.0 ± 8.0^{efghij}
	DD		N/A	51.6 ± 12.8^{hijklmnop}
AMMONIA PRETREATMENT AT 40°C	SW	2.5	80.3±3.6	43.6 ± 6.0^{efghijk}
	AA		79.8± 3.2	37.6 ± 7.3^{efghijklmn}
	SH		65.4 ±4.9	44.1 ± 3.0^{efghijk}
	BG		70.2 ± 6.7	15.7 ± 2.8^{p}
	SW	5	80.3±3.6	40.9 ± 4.0^{cdef}
	AA		79.8± 3.2	44.5 ± 5.7^{efghijk}
	SH		65.4 ±4.9	72.4 ± 2.4^{ab}
	BG		70.2 ± 6.7	41.4 ± 3.5^{efghijkl}
AMMONIA PRETREATMENT AT 60°C	SW	2.5	80.3±3.6	38.8 ± 1.7^{efghijklm}
	AA		79.8± 3.2	31.3 ± 3.9^{hijklmnop}
	SH		65.4 ±4.9	52.0 ± 2.1^{cdefg}
	BG		70.2 ± 6.7	37.9 ± 4.8^{efghijklmn}
	SW	5	80.3±3.6	72.4 ± 2.3^{ab}
	AA		79.8± 3.2	71.6 ± 4.5^{ab}
	SH		65.4 ±4.9	74.2 ± 4.3^{ab}
	BG		70.2 ± 6.7	65.1 ± 2.8^{bc}
AMMONIA PRETREATMENT AT 80°C	SW	2.5	62.3 ± 3.7	33.1 ± 7.1^{ghijklmnop}
	AA		68.3 ± 4.2	25.7 ± 4.0^{klmnop}
	SH		64.1±1.9	40.9 ± 5.8^{efghijklm}
	BG		54.8±3.4	30.9 ± 1.3^{hijklmnop}
	SW	5	62.3 ± 3.7	53.6 ±7.0^{cde}
	AA		68.3 ± 4.2	46.6 ± 20.8 ^{hijklmnop}
	SH		64.1±1.9	64.1 ± 8.7^{bc}
	BG		54.8±3.4	65.1 ± 2.8^{bc}
AMMONIA PRETREATMENT AT 121°C	SW	2.5	53.8± 3.9	26.3 ± 6.5^{jklmnop}
	AA		43.5 ± 4.0	24.1 ± 6.2^{lmnop}
	SH		40.2± 7.8	33.2±25.0 ^{efghijklmnop}
	BG		42.1 ± 5.6	17.6 ± 1.6^{op}
	SW	5	53.8± 3.9	27.6 ± 6.8^{ijklmnop}
	AA		43.5 ± 4.0	31.7 ± 7.0^{hijklmnop}
	SH		40.2± 7.8	43.9 ± 11.4^{efghijk}
	BG		42.1 ± 5.6	19.8 ± 1.1^{nop}
ULTRASONICATION 1 MIN	SF	2.5	89.4 ± 3.4	48.6± 14.9^{cdefgh}
	DD		90.3 ±1.5	19.2 ± 2.9^{nop}
	SF	5	89.4 ± 3.4	83.2 ± 1.4^{a}
	DD		90.3 ±1.5	49.9±20.0^{cdefgh}
ULTRASONICATION 3 MIN	SF	2.5	90.4 ± 3.6	49.8 ± 9.3^{cdefgh}
	DD		89. 5 ± 2.3	18.5 ± 2.1^{op}

	SF	**5**	**90.4 ± 3.6**	**49.8 ± 9.3[a]**
	DD		**89. 5 ± 2.3**	**18.5 ± 2.1[efghi]**

Table 3

	Sugar Content (mg/g biomass)				
Pretreatment	Glucose	Xyglose	Arabinose	Galactose	Mannose
CSW60	338.5 ± 10.5	186.5 ± 8.0	7.4 ± 0.5	0	0
CAA60	304.5 ± 14.3	130.5 ± 8.1	29.9 ± 2.2	0	0
CSH60	292.8± 51.7	252.1±11.8	59.9 ± 5.2	213.2.± 13.5	0
USF3	471.5±6.3	1.9 ± 0.0	234.7± 13.5	0	0
USDD3	222.4 ± 2.5	45.4±3.3	21.6 ± 2.9	0	0
CSBG3	275.2 ± 8.3	128.6 ± 6.7	1.6 ± 0.0	0	0
UTSW	275.2± 24.9	117.9 ± 3.9	9.9 ± 1.7	0	0
UTAA	345.2 ± 10.7	68.2 ± 2.9	1.6 ± 0.8	0	89.2 ± 13.4
UTSH	291.8 ± 8.4	1.9 ± 0.0	48.2 ± 4.8	210.9 ± 4.8	0
UTSF	215.6 ± 12.8	43.1 ± 2.8	31.6 ± 4.1	0	0
UTDD	319.6 ± 25.6	0	132.6 ± 8.1	0	0

CSW60, CAA60, CSH60, CBG60, USF3 & UDD3 are combined optimized pretreatment hydrolyzates of the biomass.
UTSW, UTAA, UTSH, UTSF and UTDD are unpretreated controls.

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