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Research Article

Synthesis of Biopolymers from a *Geobacillus* sp. WSUCF1 Using Unprocessed Corn Stover

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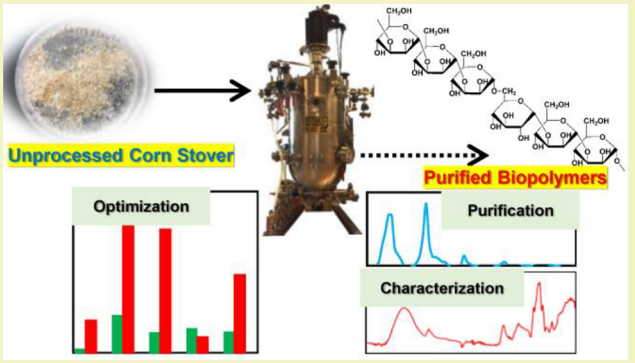
ABSTRACT: A lignocellulolytic and thermophilic bacterium *Geobacillus* sp. WSUCF1 was investigated for its production of biopolymers (exopolysaccharides, EPSs) using agricultural waste corn stover. The maximum EPS production achieved was 410 mg/L in a 40-L bioreactor. Four purified EPSs were obtained: the two neutral EPSs were glucomannan and the two negatively charged EPSs were mannan. The molecular weight of all four EPSs was estimated to be approximately 1000 kDa, and their FTIR and NMR spectra indicated that they were mainly composed of an α-type glycosidic bond in a linear structure. The mannan EPSs had a low level of crystallinity and displayed high thermal stability, with thermal degradation temperatures of 309 and 316 °C, while the glucomannan EPSs were essentially amorphous and had moderate thermal stability, with thermal degradation temperatures of 203 and 227 °C. A mannan EPS (EPS 1−2) showed exceptional biocompatibility, with a noncytotoxic concentration as high as 4000 μg/mL using HEK-293 cell line. The green metrics showed that EPS production using corn stover appears to be more sustainable than using glucose. This study reports for the first time a cultivation strategy for EPS production by a lignocellulolytic thermophile using corn stover as a carbon source, requiring no biomass pretreatment.



KEYWORDS: *Thermophile, Geobacillus, Exopolysaccharide, Corn stover, Thermostability, Bioactivity, Green metrics*

INTRODUCTION

Over the last two decades, extremophilic bacteria have been explored as unique resources for various kinds of bioproducts, such as biofuels, thermostable enzymes, and biopolymers.[1] The extremes of temperature, salinity, and environmental pH usually endow outstanding production capabilities to the extremophiles and nontraditional properties to their products. Exopolysaccharides (EPSs), an important type of natural and microbially produced polymer, have been investigated using different kinds of extremophilic bacteria to obtain novel molecular structure and stronger bioactivities compared with more common carbohydrate polymers.[2] Among those extremophilic EPS-producers, thermophilic bacteria perform as nonpathogenic microorganisms, and their short period of EPS productivity can be appealing as potential cell factories for these value-added natural products.[3]

The fermentation of thermophilic bacteria usually requires using sugar substrates as carbon sources for biomass growth and accumulation of bioproducts, especially for those energy-intensive bioprocesses such as EPS production. However, production processes which rely on costly pure sugars hamper their large-scale industrial application.[4] In addition to offering unique properties, thermophilic EPSs should be cost competitive with commercialized carbohydrate polymers obtained from plant or mesophilic bacteria resources. The fermentation medium is considered to represent around 50% of the cost for a fermentation process, and the utilization of inexpensive and renewable substrates as substitutes for monosaccharide or disaccharide carbon sources is an attractive route to more economical bioprocessing and production of EPS.[5]

Byproducts from some other industries can provide promising substrates for cost-effective fermentation processes. For example, the molasses byproduct from the sugar industry retains high sugar content and could be applicable as an inexpensive carbon source for the production of mesophilic EPSs such as xanthan,[6] gellan,[6] pullulan,[7] and cellulose.[8] Besides mesophiles or neutrophiles, a halophilic bacterium *Halomonas* sp. AAD6 successfully utilized pretreated molasses as a replacement for sucrose to produce a levan-type EPS which demonstrated high biocompatibility[5] and flocculating

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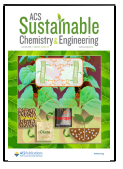

activity.[9] Agricultural waste can also provide potential substrates for EPS production. The production of dextran by mesophilic bacterium *Leuconostoc mesenteroides* using carob pod extract has been achieved with high productivity since carob pod has a high content of sucrose.[10] Both levan and dextran are homopolysaccharides, and their biosynthesis takes place extracellularly through levansucrase and dextransucrase, respectively. The thermophilic EPSs are generally heteropolysaccharides and their biosynthesis requires complex intracellular enzymatic systems for precursors, assembly, export, and regulation.[11]

Agricultural waste in the form of nonfood plant biomass is considered to be almost inexhaustible, and this type of second-generation lignocellulosic feedstock has already been studied for biofuel production by different thermophilic bacteria.[1] The fermentation of lignocellulosic substrates usually requires a primary pretreatment process to release more fermentable composition from their recalcitrant backbone which significantly increases the bioprocessing cost.[12] Recently, thermophilic *Geobacillus* species have been studied for their uncommon thermostable lignocellulolytic enzymes which could be incorporated in a consolidated bioprocess combining enzymatic hydrolysis of lignocellulose and fermentation utilizing the hydrolysates.[13] These thermophilic lignocellulose-degrading bacteria would facilitate the development of a more cost-effective fermentation process to convert lignocellulosic biomass into biofuels and other value-added bioproducts.[14]

Geobacillus sp. WSUCF1 is a thermophilic bacterium which possesses a complete repertoire of lignocellulose deconstruction enzymes specific to the hydrolysis of cellulose and hemicellulose.[11] It was found that it can grow by utilizing inexpensive unprocessed lignocellulosic biomasses such as prairie cord grass and corn stover to produce a cellulase[14] and xylanase cocktail with substantial activity and thermostability.[15] In our former study using glucose as carbon source, the strain WSUCF1 was found to produce two different EPSs, which were glucomannan and mannan, with high thermostability and biocompatibility (unpublished results). The aim of this study is to explore the cultivation for EPS production by thermophilic bacterium *Geobacillus* sp. WSUCF1 using unprocessed lignocellulosic substrates as carbon and energy sources. The concise green metrics analysis suggests that the EPS production by strain WSUCF1 using a renewable lignocellulosic substrate can be a more economically viable bioprocess for biopolymer manufacture. We also explore some of the properties of the EPSs synthesized from using a complex lignocellulosic substrate, including biocompatibility, antioxidant activity, and thermal stability, since they may demonstrate significant industrial potential.

EXPERIMENTAL SECTION

Microorganism and Culture Conditions. *Geobacillus* sp. strain WSUCF1 used in this study was isolated from soil sample obtained from a compost facility at Washington State University, Pullman, WA.[14] It was routinely cultivated in agar plate (20 g/L agar) containing (g/L) glucose 6.0, yeast extract 1.0, and NaCl 3.0 at pH 7.0 and 60 °C for 24 h.

EPS Production Using Lignocellulosic Biomasses. To study the effect of unprocessed lignocellulosic biomass on EPS production by the *Geobacillus* sp. strain WSUCF1, corn stover, prairie cord grass, ponderosa pine, and switch grass were used as carbon and energy sources without any chemical or enzymatic pretreatment having been applied to these lignocellulosic substrates. The basal medium for EPS production by strain WSUCF1 contained (g/L): lignocellulosic carbon source, 6.0; yeast extract, 1.0; and NaCl, 3.0. The initial pH was adjusted to 7.0 by 1 M NaOH. The medium was sterilized at 121 °C for 15 min. For each assay of EPS productivity, strain WSUCF1 was inoculated from an agar plate and grown in a 250 mL Erlenmeyer flask with 50 mL corresponding liquid medium and cultivated in a shaking incubator at 60 °C, 180 rpm for 24 h as seed culture. Then seed culture was inoculated (5%, v/v) into Erlenmeyer flask of 1000 mL containing 300 mL liquid medium and allowed to grow for 24 h at 60 °C and 180 rpm.

Using the most favorable lignocellulosic carbon source for EPS production, the EPS production was further optimized step-by-step in the following order: concentration of nitrogen source, concentration of carbon source, and concentration of NaCl. At each step the optimized conditions obtained from the previous steps were retained. EPS and cell growth were measured according to the analytical methods, and all experiments were carried in triplicates.

EPS Production in Bioreactor. The scale-up of EPS production by strain WSUCF1 was performed in a 40-L bioreactor (New Brunswick BioFlo 510 Fermentor, Eppendorf, Inc., Hauppauge, NY, U.S.A.) with a working volume of 20 L using optimized liquid medium (g/L): corn stover 6.0, yeast extract 15.0, and NaCl 3.0 (pH 7.0) sterilized in situ. The fermentation cultivation in the bioreactor was inoculated with a 5% (v/v) of seed culture of strain WSUCF1. During the EPS-producing process, temperature was maintained at 60 °C, and the pH of the medium was monitored and left natural. The bioreactor was operated in batch mode and aerated at 2 vvm with constant agitation. Bacterial growth and EPS accumulation were measured by sampling 20 mL of culture broth.

Analytical Methods. The cell growth was measured by optical density (OD) at 600 nm and transferred to dry cell weight by a calibration curve of cell concentration versus OD_{600}. The insoluble corn stover particles were allowed to settle for 20 min before the OD test.[16] Afterward, 100 μL fermentation broth without insoluble corn stover was added into a 96-well microtiter plate, and the absorbance was measured at 600 nm using a microplate reader (EPOCH 2, BioTek, Winooski, VT, U.S.A.). The bacterial cells were removed by centrifugation at 6000*g* for 15 min, and EPS in cell-free supernatant was precipitated by adding equal volume of chilled absolute ethanol. The alcoholic mixture was kept at −20 °C overnight and then the EPS pellet was collected by centrifugation at 8000*g* for 40 min. The carbohydrate content was measured by the phenol-sulfuric acid method using glucose as standard.[17] The protein content was evaluated through the Bradford method with bovine serum albumin as standard.[18] The nucleic acid content was measured by the absorption at A_{260}. The same procedure was also performed using uninoculated medium as control. The carbohydrate, protein, and nucleic acid contents in EPS were calculated subtracting the corresponding values of blank medium when necessary. Uronic acid content was estimated by the method in Blumenkrantz[19] using galacturonic acid as standard. The metabolic byproducts were analyzed in cell-free culture supernatant using HPLC as described previously.[20] The activity of the cellulose-degrading enzyme of strain WSUCF1 during EPS production using corn stover as substrate was estimated according to the method in Wang.[20] The xylanase activity was analyzed by the method in Bhalla[15] at a reaction temperature of 60 °C.

Isolation and Purification of EPS. The proteins in the cell-free fermentation broth were first removed by ammonium sulfate (100% saturation) precipitation and centrifugation at 10 000*g* for 30 min. Then an equal volume of chilled absolute ethanol was added to the supernatant and the solution was kept at −20 °C overnight. The precipitated EPS was harvested by centrifugation at 8000*g* for 40 min and dialyzed against deionized water for 3 days.

The crude EPS was further purified using anion-exchange chromatography with a DEAE Sepharose CL-6B column (1.9 × 60 cm^2). The crude EPS was eluted with 100 mL deionized water at a flow rate of 0.2 mL/min, and then with a discrete NaCl gradient from 0.1 to 1.0 M with an increment of 0.1 M (100 mL for each concentration). The elution fractions (10 mL for each fraction) were analyzed by phenol-sulfuric acid method. EPS contained fractions

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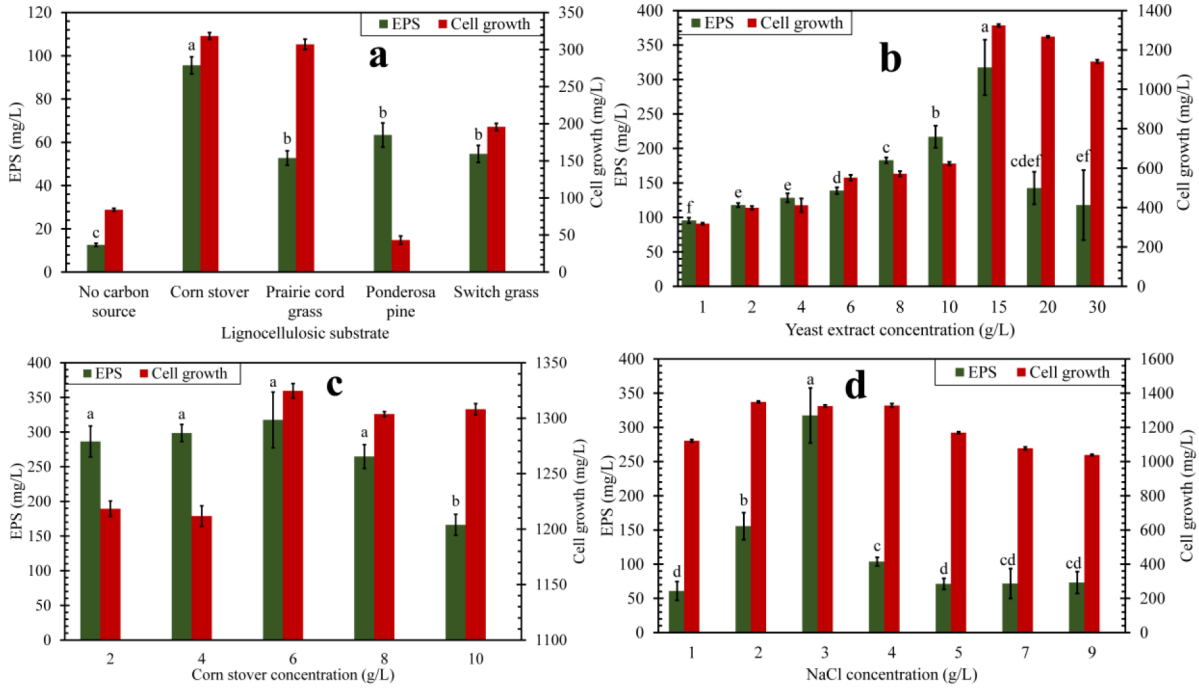

Figure 1. Effect of (a) untreated lignocellulosic substrates, (b) yeast extract concentration, (c) corn stover concentration, and (d) NaCl concentration on the growth and EPS production by *Geobacillus* sp. WSUCF1. Different letters above each column indicate statistical differences of EPS production among groups at $p < 0.05$.

were combined, concentrated by rotary evaporator, and then eluted using Sephadex G-50 column (2.5 × 50 cm^2) with deionized water as eluent with a flow rate of 0.5 mL/min.

EPS Characterization. *Monosaccharide Composition Analysis.* The purified EPS was hydrolyzed by 2 M trifluoroacetic acid (TFA) at 120 °C for 2 h in a sealed vial and then vacuum-dried. The hydrolysate was applied for the quantification of the constituent monosaccharide monomers using HPLC (Shimadzu LC20, Columbia, MD, U.S.A.), equipped with Aminex HPX-87H column (300 × 7.8 mm^2 inner diameter) and refractive index detector (RID). The column temperature was 50 °C, with 5.0 mM H_2SO_4 as eluent, at a flow rate of 0.4 mL/min. The relative molar ratio was calculated by the area normalization method. Monosaccharide was identified by comparison with standard monosaccharides. Pyruvic acid content was analyzed by the method in Anthon.[21] The presence of sulfate was tested by the method in Silvestri.[22]

Molecular Weight. The purified EPS was eluted by gel filtration on Sepharose CL-6B column (1.9 × 80 cm^2) with deionized water as eluent at a flux of 0.1 mL/min. Each fraction (2 mL) was analyzed by phenol-sulfuric acid method. A group of dextrans (150, 270, 410, 670, and 1100 kDa) were applied as standards and eluted at the same conditions. The molecular weight of the EPS was derived from the standard plot of the dextran standards.

FTIR and NMR. FTIR characterization of EPS was performed using Agilent Cary 600 FTIR spectrometer (Agilent Technologies, Santa Clara, CA, U.S.A.). The EPS was mixed with KBr in a ratio of 1:50 (w/w) and pressed into a pellet for FTIR analysis. NMR spectra of EPS were recorded at 300 (^{1}H) or 75 MHz (^{13}C) on a Bruker Avance-II 300 spectrometer (Bruker BioSpin, Karlsruhe, Germany) in D_2O (40 mg/mL) at room temperature. Chemical shifts were reported in parts per million (ppm) with reference to acetone for ^{1}H and ^{13}C NMR spectra.

Thermogravimetric Analysis and X-ray Diffraction Analysis. The thermogravimetric analysis (TGA) was recorded with a TA Q600 TGA apparatus (TA Instruments Inc., New Castle, DE, U.S.A.), and EPS specimens were scanned from 20 to 600 °C at a heating rate of 10 °C/min under nitrogen atmosphere with a flow of 20 mL/min. X-ray diffraction (XRD) was analyzed by an Ultima-Plus X-ray powder diffractometer (Rigaku Co. Ltd., Tokyo, Japan). The ground EPS sample was placed on a sample holder and the XRD under CuKα irradiation was tested at room temperature. The diffractometer was operated at 40 kV and 40 mA. The diffractogram was scanned at 2θ angles between 3° to 60° at a rate of 2°/min. The d-spacing at the value of θ was calculated by Bragg's law, and crystallinity index was calculated from the area under crystalline peaks normalized to the total scattering area.

Cytotoxicity Assay. Human embryonic kidney 293 (HEK-293) cells (ATCC CRL-1573) were kindly provided by Dr. Jing Liu, Indiana University–Purdue University Indianapolis, Indianapolis, IN, U.S.A. HEK-293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 50 μg/mL penicillin, and 50 μg/mL streptomycin at 37 °C in an atmosphere of 5% CO_2. For the cytotoxicity assay, 200 μL media containing 8 × 10^3 HEK-293 cells per well were seeded into a 96-well microtiter plate which was then incubated at 37 °C in an atmosphere of 5% CO_2 for 24 h. Afterward, the culture supernatant was carefully discarded, and the cells were further exposed to EPS dissolved in DMEM and incubated for 24 h at 37 °C in an atmosphere of 5% CO_2. The (3-(4,5-dimethylthiazol-2-yl)-2,5-diphynyltetrazolium bromide (MTT) assay was applied for the quantification of live cells. DMEM without EPS was used as negative control which was considered 100% viable. All the experiments were performed in triplicate.

Antioxidancy Assay. The free radical scavenging activities of purified EPS against hydroxyl radical (•OH), 1,1-diphenyl-2-picryl-hydrazyl radical (DPPH$^•$) and superoxide anion ($O^{2-•}$) were estimated according to the methods in Sun.[23]

Green Metrics Calculations. The green metrics were calculated according to the methods in Pinazo.[24] Parameters including *E*-factor, product-to-input ratio, material efficiency, energy efficiency, land use, and material cost were considered and compared between the EPS producing processes using glucose or corn stover as a carbon source. The unit mass to produce EPS applied in the calculation was 1 kg. The parameters used in green metrics analysis are given in the online Supporting Information (SI).

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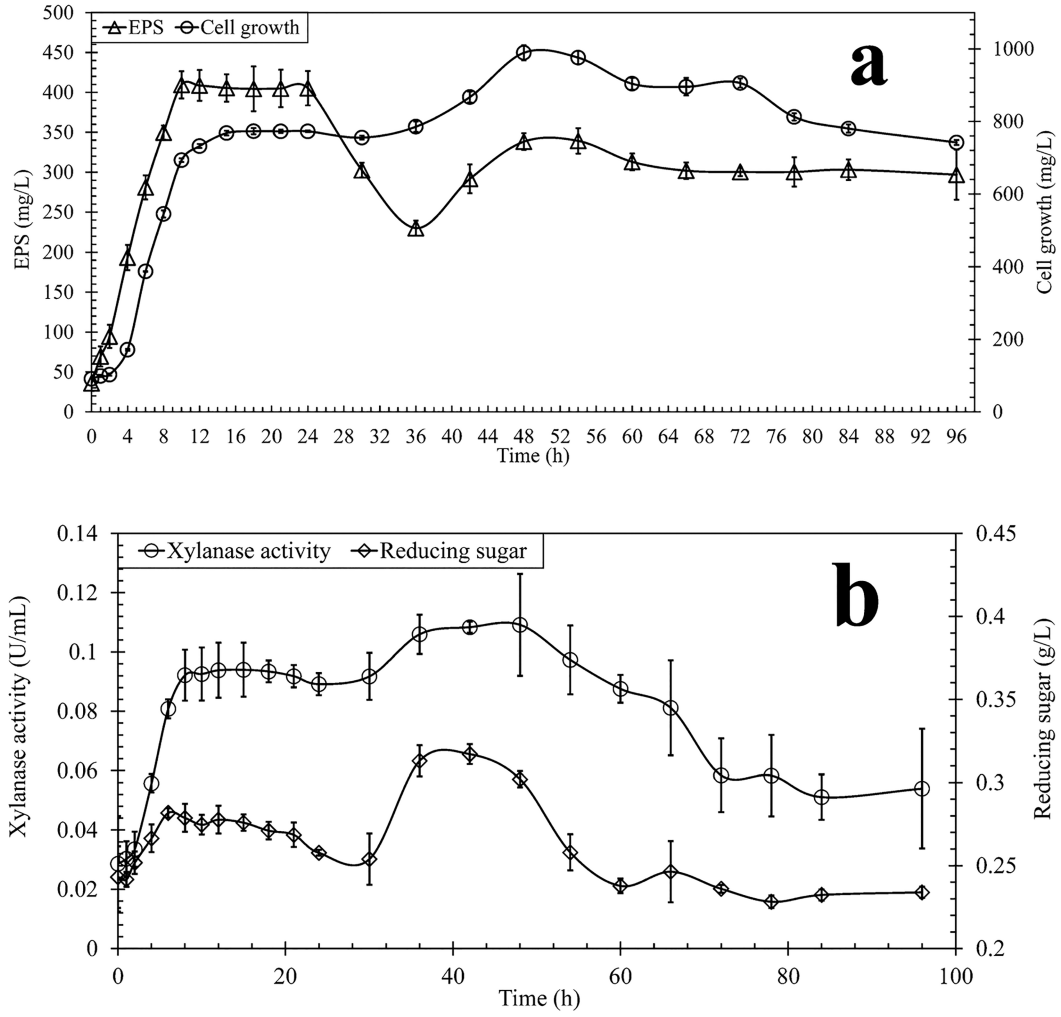

Figure 2. Time course of (a) growth and EPS production and (b) reducing sugar content and xylanase activity of *Geobacillus* sp. WSUCF1 using corn stover as carbon source in bioreactor. Values are means with standard error bars, and error bars smaller than the symbols are not shown.

Statistical Analysis. The experimental data were expressed as mean value ± standard error calculated from three parallel experiments. The statistical analysis was performed by one-way analysis of variance (ANOVA) using Microsoft Excel. Differences were regarded as significant when $p < 0.05$.

RESULTS AND DISCUSSION

Optimization of EPS Production Using Lignocellulosic Biomass. To investigate the effect of lignocellulosic carbon sources on the growth and EPS production of thermophilic strain WSUCF1, 4 different lignocellulosic biomasses (corn stover, prairie cord grass, ponderosa pine, and switch grass) were provided at 6 g/L, instead of employing glucose as the carbon source in the basal medium. It was found that strain WSUCF1 could grow on a wide range of lignocellulosic biomass, and the highest amount of cell growth and EPS production (95.56 mg/L) was achieved in the corn stover medium (Figure 1a). An intriguing phenomenon is that high cell growth does not always result in a correspondingly higher EPS production level for strain WSUCF1. When using prairie cord grass, the carbon flux might not direct to nucleoside diphosphate saccharide (NDP-sugar) precursor synthesis. This phenomenon could arise from low activity of critical enzymes at the principal branch point to NDP-sugar synthesis. Furthermore, different lignocellulosic carbon sources may have different catabolic repression effects on the cellular secondary metabolism.[25,26] The observed production of EPS from unprocessed lignocellulosic biomass demonstrates the industrial potential of strain WSUCF1, and the use of corn stover as substrate for EPS biosynthesis could be promising as the basis of an economical and eco-friendly bioprocess. In the light of these results, unprocessed corn stover was selected as the preferred carbon source for the following study.

Although a lot of appealing properties have been revealed for thermophilic EPSs, the EPS producing capability by thermophilic bacteria is still relatively inadequate compared with mesophilic EPS producers.[4,27] A basic approach to maximizing EPS production is to optimize the extrinsic factors, since the amount of EPS production can greatly depend on the composition of the culture medium.[28,29] The effect of yeast extract on EPS production by strain WSUCF1 was examined since, when using a sugar carbon source, the yeast extract concentration has been found to be a crucial factor to increase EPS productivity (unpublished results). When using corn stover as carbon source, the optimum yeast extract for both bacterial growth and EPS production was 15 g/L (Figure 1b). A high yeast extract concentration might be advantageous for

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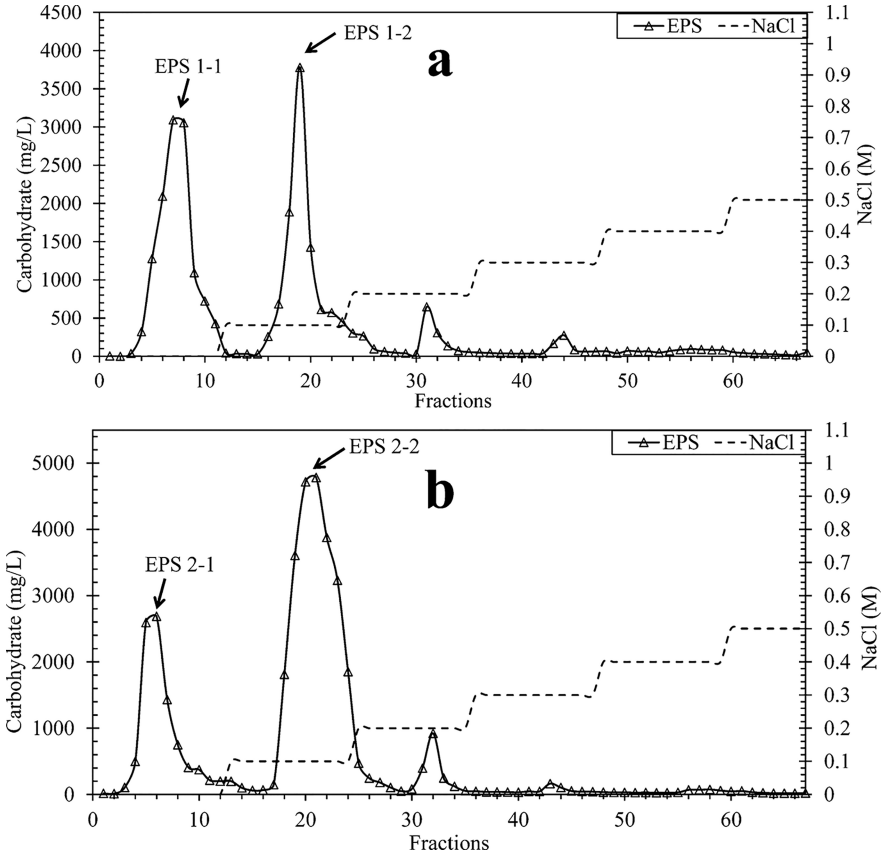

Figure 3. Purification profile of (a) Crude EPS 1 and (b) Crude EPS 2 of *Geobacillus* sp. WSUCF1 on DEAE Sepharose CL-6B column.

strain WSUCF1 to degrade a lignocellulosic substrate and more energy may be provided for the biosynthesis of EPS as a secondary metabolite.

The role of yeast extract in EPS production remains vague due to the use of different strains. For example, *Xanthomonas*, *Pseudomonas*, and *Rhizobium meliloti* produced higher amounts of EPS in nitrogen-limiting conditions.[2,30] However, Gorret[31] reported that the addition of yeast extract to the medium could directly improve both growth and EPS production by *Propionibacterium acidipropoinici*. Another mesophilic bacterium *Bordetella* sp. B4 produced a glucan type EPS in the medium containing 7.5 g/L glucose, 20 g/L yeast extract and several other mineral salts.[32] For thermophilic EPS producers, the stress which motivates EPS production is from extreme temperature. Therefore, no additional type of stress besides the extreme temperature is required to stimulate EPS biosynthesis for thermophiles. The nutrient can be enriched to provide a favorable environment for extremophiles to produce EPSs in the extremophilic conditions, and no limitation on the availability of nutrients is necessary for EPS overproduction by extremophiles.

The effect of the corn stover concentration was sequentially analyzed and a favorable corn stover concentration for EPS production by strain WSUCF1 was found to be 6 g/L (Figure 1c). When the corn stover concentration was 10 g/L, the EPS production was much lower than that at the optimum corn stover concentration. The fact that higher content of corn stover in the medium appears disadvantageous for EPS biosynthesis might be due to the rising content of fermentation inhibitor in the corn stover, released during enzymatic hydrolysis.[33] It was then found that the optimum NaCl concentration for EPS production was 3 g/L (Figure 1d). When the NaCl concentration was higher than 3 g/L, the EPS production was significantly inhibited. For the nonhalophilic strain WSUCF1, a higher level of NaCl concentration could not enhance the production of EPS, suggesting no protective response by the bacterium. In summary, when using corn stover as carbon source, strain WSUCF1 could produce 317.5 mg/L EPS on the optimized culture medium which was comparable with what we obtained using glucose (unpublished results).

EPS Production Scale-Up in Bioreactor. Scale-up from bench to pilot scale fermentation, once optimal conditions were identified, is required to produce the EPS in suitable volume. For EPS-production scale-up, a 40-L fermenter with 20 L working volume was used with the optimized culture medium. The WSUCF1 strain revealed interesting features in growth and EPS production, as shown in Figure 2a. Both growth and EPS production exhibited two phases. The first phase took place from 0 to 24 h, with a maximal EPS production of 409.5 mg/L at 10 h, and a specific EPS yield of 0.53 g EPS/g cell dry weight. In the first phase, the EPS production demonstrated a concomitant rise with cell growth, suggesting a growth associated status. Growth association of EPS production appears common for extremophilic EPS, but it does not occur with some of the mesophilic EPS producers, possibly because the protecting effect of EPS is not required during their growth.[34] For thermophilic EPS producing processes, the maximum amount of EPS production is usually reached in significantly shorter times than those of mesophilic EPS producers.[3,35] For instance, thermophilic EPS producer *Geobacillus thermodenitrificans* ArzA-6,[35] *G. toebii* ArzA-8,[35]

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Table 1. Chemical−Physical Characterization of EPSs Produced by *Geobacillus* sp. WSUCF1 Using Corn Stover

properties	EPS 1−1	EPS 1−2	EPS 2−1	EPS 2−2
carbohydrate content (%, w/w)	94.6 ± 2.6	93.9 ± 2.7	94.9 ± 2.8	94.4 ± 0.9
protein content (%, w/w)	2.3 ± 1.1	4.0 ± 0.3	2.3 ± 0.1	2.9 ± 0.0
nucleic acid content (%, w/w)	1.2 ± 0.0	0.5 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
uronic acid (%, w/w)	1.9 ± 0.4	1.6 ± 0.1	2.7 ± 0.2	2.6 ± 0.1
monosaccharide composition (by molar ratio)	mannose/glucose (1/0.73)	mannose	mannose/glucose (1/0.18)	mannose
pyruvic acid (%, w/w)	2.0 ± 0.6	0.2 ± 0.1	2.1 ± 0.5	0.1 ± 0.1
molecular weight (kDa)	∼1000	∼1000	∼1000	∼1000
degradation temperature (°C)	203	316	227	309

Aeribacillus pallidus 418,[34] and *Brevibacillus thermoruber* 423[3] attained maximum EPS product amount at 12 h. For another thermophilic bacterium *Anoxybacillus tepidamans*, the maximum quantity of EPS was reached after only 8 h of cultivation.[36,37] Mesophilic biopolymer synthesis usually takes place after the medium is depleted of one or more nutrients, but for thermophilic strains the stress from the harsh environment can directly induce the biopolymer synthesis process, which results in a relatively short fermentation process for biopolymer production.

However, from 24 to 36 h the EPS content dropped to a lower level, while the bacterial cell level remained stable. After 36 h, both EPS accumulation and cell growth increased for a second time. The second phase of cell growth could be due to the further degradation of corn stover by the lignocellulolytic enzymes produced by strain WSUCF1, leading to a supplement of utilizable carbon source. Meanwhile, the decreasing of EPS content might also be related to the degrading effect by those enzymes. In a former study, EPS degradation took place with prolonged incubation, and it was speculated to be degraded by the glycohydrolase generated by the strain itself.[38] Using simple sugar as carbon source may not activate transcription of the enzymes for degradation of EPS. According to this two-phase EPS producing profile, the crude EPSs were collected from these two EPS accumulation peaks as Crude EPS 1 (24 h) and Crude EPS 2 (54 h), and they were both further purified.

During the EPS production process, strain WSUCF1 showed no significant CMCase activity, indicating it might not utilize the cellulose component from the corn stover substrate. The profile of xylanase activity was similar to that of the growth of strain WSUCF1, which had a second phase of increase at 30 to 48 h (Figure 2b). Meanwhile, the content of reducing sugar was also accumulated at the same time range due to the higher xylanase activity. The consumption of reducing sugar during the exponential phase was substantial since the EPS production by thermophilic strain WSUCF1 is growth-associated. The cellulase transcription of strain WSUCF1 might not be activated during EPS production using the corn stover substrate. In the former report, it was shown that strain WSUCF1 could generate CMCase activity when using unprocessed prairie cord grass as carbon source.[14] In future work, for the improvement of EPS yield, the prairie cord grass could be supplemented with corn stover to generate the enzymes capable of degrading the cellulose component in the lignocellulosic substrate. This may also be a strategy for the production of EPSs with various monomer ratios, leading to different engineered properties with industrial potential.

Purification of EPS. Both crude EPSs contained more than 80% (w/w) carbohydrate. The purification of Crude EPS 1 and 2 had a similar profile with two major carbohydrate peaks (Figure 3a and 3b). In both cases, the first EPS peak (EPS 1−1 and EPS 2−1) was a neutral polysaccharide while the second EPS peak (EPS 2−1 and EPS 2−2) was a negatively charged polysaccharide. These elution results were also identical to that from the crude EPS obtained using glucose as carbon source (unpublished results). This consistency of EPS fractions produced by strain WSUCF1 from glucose and corn stover indicate that the EPS biosynthesis in strain WSUCF1 might be administrated by the same EPS gene clusters, and the mechanisms of EPS biosynthesis when using different carbon substrates could be identical. The minor peaks at higher concentration of NaCl had low content of carbohydrate (less than 10% w/w) and they were not further studied. The major peaks were further purified using gel filtration column, respectively. Each EPS fraction exhibited only one peak suggesting all of them were at qualified purify for characterization. These purified EPSs were assigned as EPS 1−1 (neutral EPS from Crude EPS 1), EPS 1−2 (negatively charged EPS from Crude EPS 1), EPS 2−1 (neutral EPS from Crude EPS 2), and EPS 2−2 (negatively charged EPS from Crude EPS 2) in the following investigation of characteristics.

EPS Characterization. *Monosaccharide Composition.* All 4 purified EPSs were further analyzed for their physicochemical properties. The total carbohydrate content for each EPS was around 94% (w/w), suggesting that the main component of the EPSs was polysaccharide (Table 1). Both negatively charged EPSs showed 2.9 to 4% (w/w) protein content. The relatively higher content of nonsugar moieties such as protein may endow the negative charge character to the EPSs.[39] Further analysis revealed the presence of pyruvate as an organic substituent in all EPSs. The monosaccharide component of these neutral and negatively charged EPSs were identical with those of the corresponding EPSs from glucose, while the molar ratio between mannose and glucose monomers were different (unpublished results). All EPSs had the same molecular weight, indicating that their molecular weight might be controlled by the same proteins for EPS chain-length determination. The NDP-sugar precursors, glycosyltransferases, and export system may be identical when using different substrates for EPS production. However, the activities of glycosyltransferases might be different, thus leading to the variation in monomer ratios. Moreover, strain WSUCF1 was putatively assigned as using ATP-binding cassette (ABC) transporter-dependent pathway for EPS secretion.[11] Due to the variety of monomer ratios of the EPSs produced by strain WSUCF1 when using different carbon sources, the ABC-transporters showed an infidelity toward the monomer ratio, which might be beneficial for providing flexibility in producing EPSs with different monomer ratios through engineering strategies.

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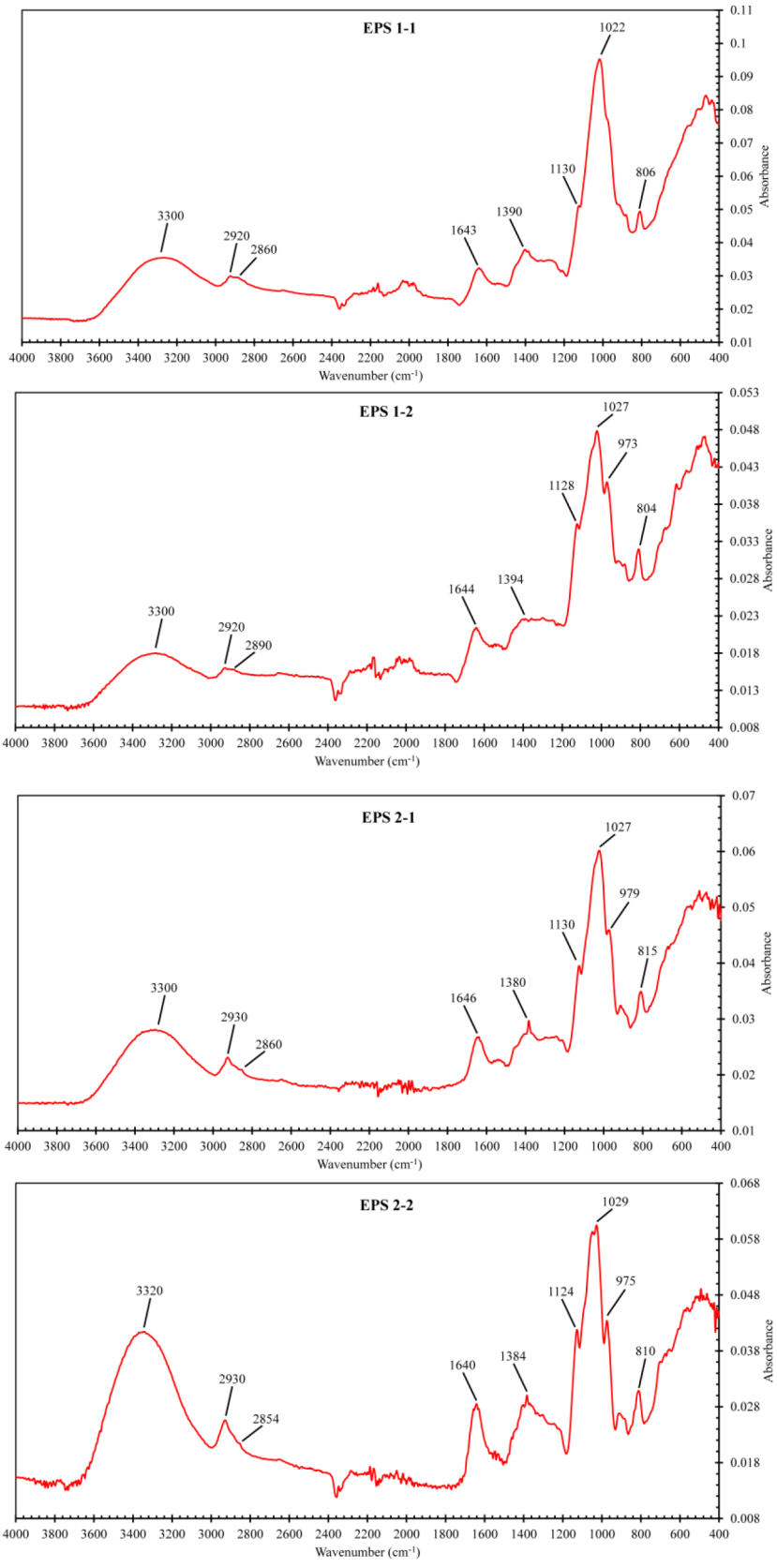

Figure 4. FTIR spectra of the EPSs produced by *Geobacillus* sp. WSUCF1 using corn stover.

Structural Analysis. The functional groups of the four purified EPSs of strain WSUCF1 were investigated through FTIR spectroscopy at the absorbance mode from 4000 to 400 cm^{-1} as shown in Figure 4, and the peaks were assigned according to literature data. Generally, the FTIR spectra of all the purified EPSs showed a pattern of FTIR absorption

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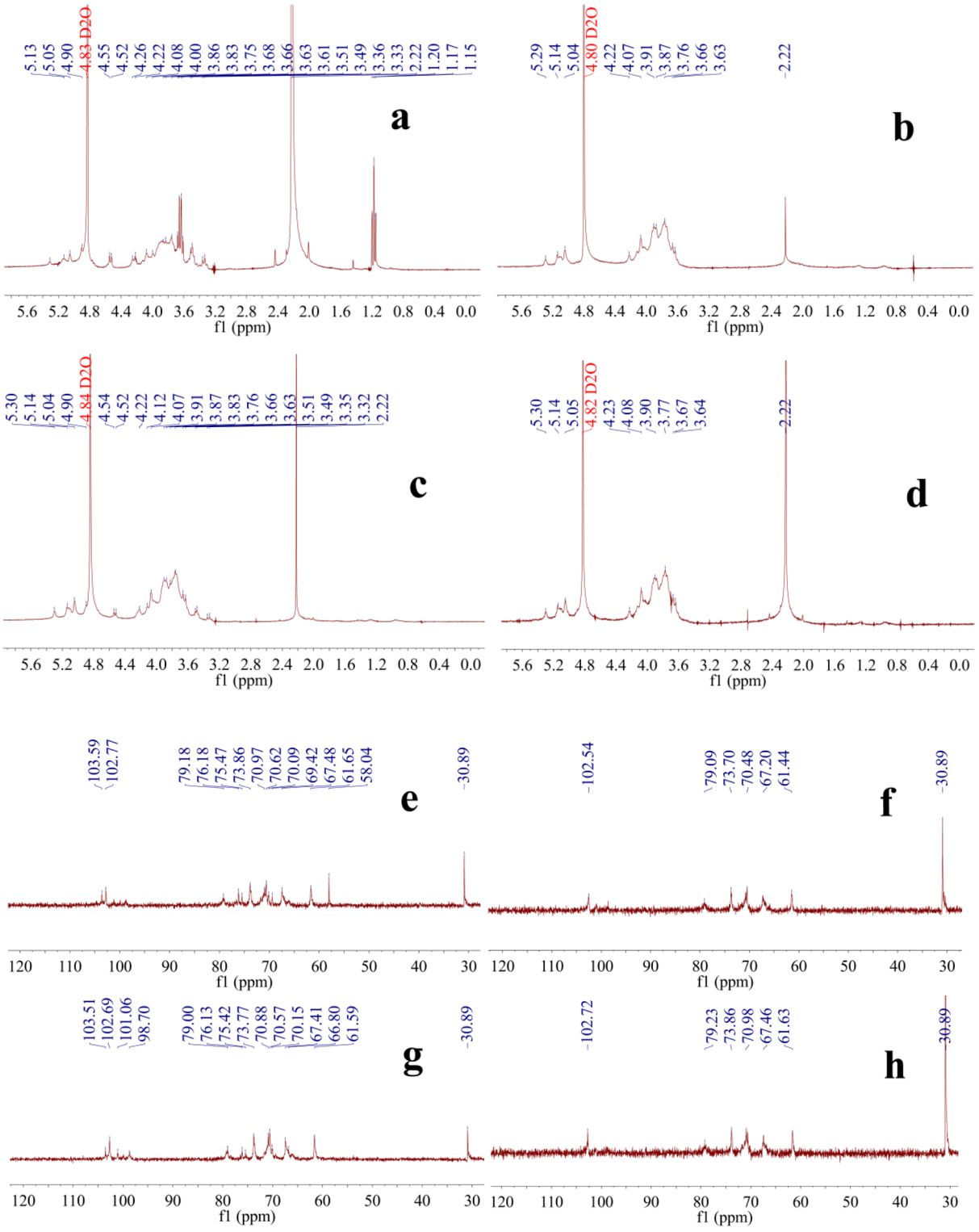

Figure 5. ^{1}H NMR spectra of (a) EPS 1−1, (b) EPS1−2, (c) EPS 2−1, (d) EPS 2−2, and ^{13}C NMR spectra of (e) EPS 1−1, (f) EPS 1−2, (g) EPS 2−1, and (h) EPS 2−2 produced by *Geobacillus* sp. WSUCF1 using corn stover.

features that are typical of polysaccharide. The broad peak at around 3300 cm^{-1} is associated with the stretching of the O−H bond. The absorption peaks at 2950 to 2850 are the asymmetrical C−H stretching bands in the pyranose ring of saccharide moieties. The signals at 1640 cm^{-1} in all the EPS spectra represent the amide I (carbonyl) bond.[40] Peaks at around 1385 cm^{-1} can be attributed to the C−H deformation oscillation.[41] The significant absorption at around 1029 to 1022 cm^{-1} frequency is the glycosidic bond C−O−C signal. Peaks with frequencies at around 900 to 800 cm^{-1} are considered to be from the anomeric region for polysaccharides, and the α-linkage is usually in the lower end of this region. Consequently, the peak appearing in the 815 to 804 cm^{-1} range indicates the presence of an α-anomeric configuration.[42]

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Table 2. Putative Assignment of Hydrogen and Carbon Chemical Shifts Based on ^{1}H and ^{13}C NMR Spectroscopic Data of EPSs Produced by *Geobacillus* sp. WSUCF1 Using Corn Stover

	chemical shifts (δ, ppm)					
	H-1 (anomeric)	H-2	H-3	H-4	H-5	H-6, H-6′
	C-1	C-2	C-3	C-4	C-5	C-6
EPS 1−1						
mannose	5.31/5.13/5.05	4.22	4.08	3.86	3.75	3.66/3.63
	103.59	70.62	79.18	69.42	73.86	61.65
glucose	4.90	4.52	4.00	3.83	3.49	3.36/3.33
	102.77	75.47	76.18	70.09	70.97	67.48
EPS 1−2						
mannose	5.29/5.14/5.04	4.22	4.07	3.87	3.76	3.66/3.63
	102.54	70.48	79.09	67.20	73.70	61.44
EPS 2−1						
mannose	5.30/5.14/5.04	4.22	4.07	3.87	3.76	3.66/3.63
	102.69	70.57	79.00	66.80	73.77	61.59
glucose	4.90	4.52	3.91	3.83	3.49	3.35/3.32
	101.06	75.42	76.13	70.15	70.88	67.41
EPS 2−2						
mannose	5.30/5.14/5.05	4.23	4.08	3.90	3.77	3.67/3.64
	102.72	70.98	79.23	67.46	73.86	61.63

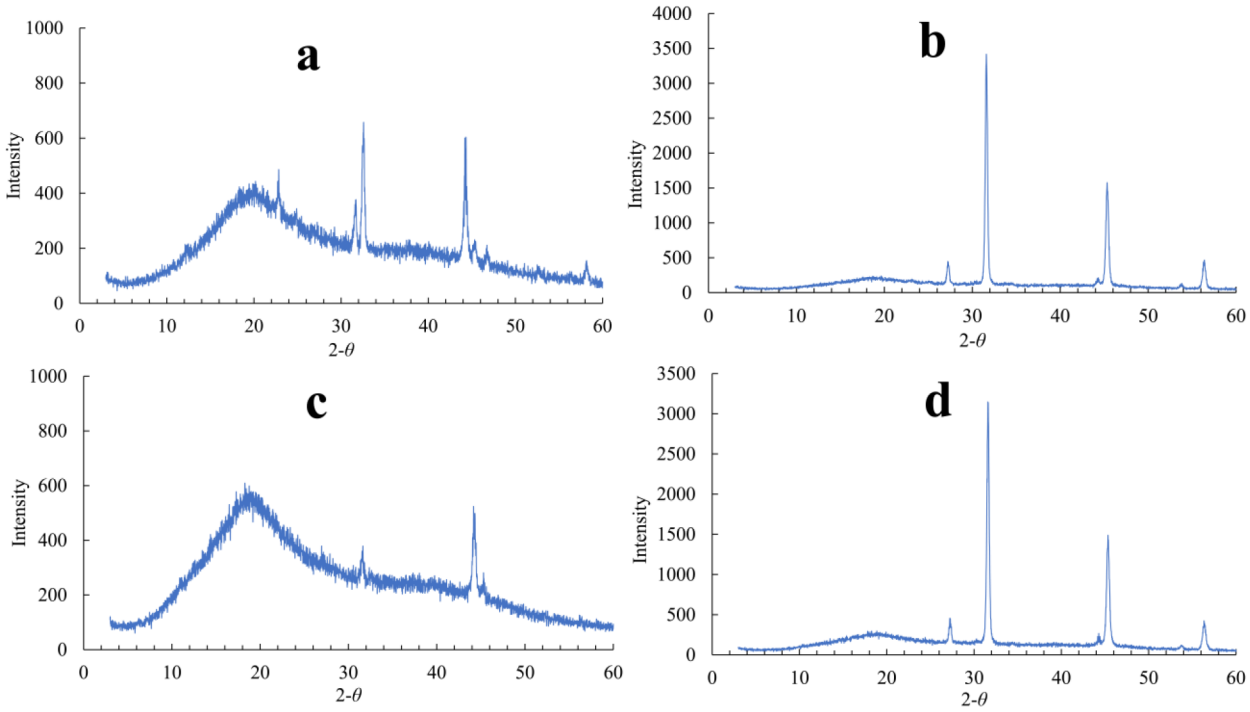

Figure 6. XRD profile of (a) EPS 1−1, (b) EPS 1−2, (c) EPS 2−1, and (d) EPS 2−2 isolated from strain WSUCF1.

The anomeric region can also be unique for each polysaccharide and provide additional information to the glycosidic bond region.[43] No obvious peak could be observed at 1260 to 1220 cm^{-1}, suggesting the absence of sulfate residues in all four EPSs.[40] The region between 1800 and 1200 cm^{-1} can be considered as a spectral signature of minor components in the polysaccharides.[44]

The structures of the four EPSs were further studied through ^{1}H and ^{13}C NMR spectra (Figure 5). The ^{1}H NMR spectra of EPSs displayed typical sugar ring resonances as the convergence of signals in a narrow region between 3.3 to 4.6 ppm. Compared with the two neutral EPSs (EPS 1−1 and 2−1), the peaks related to glucose moieties were all absent in the spectrum of negatively charged EPSs (EPS 1−2 and 2−2), only leaving signals of mannose units. The ^{1}H NMR spectrum of EPS 1−1 had extra triplet peaks at around 1.17 ppm compared with the other neutral EPS 2−1. These peaks could be tentatively assigned as methyl groups.[32] The higher peaks at δ 3.66 ppm compared with EPS 2−1 might be due to the increased content of glucose monomers in EPS 1−1, and these quartet peaks might be from the coupling effect of the adjacent methyl groups. The characteristic signals of ^{1}H NMR from EPSs were tentatively assigned to the sugar residue structures of mannose and glucose (Table 2). The ^{13}C NMR spectrum of EPS 1−1 was similar to the neutral EPS from using glucose as

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carbon source, suggesting a glucomannan-type structure with α-(1,3)-linked mannose and α-(1,6)-linked glucose monomers. The ^{13}C NMR spectrum of EPS 2−1 had two additional weak anomeric signals compared to the spectrum of EPS 1−1, which might be from trace content of β-configuration monosaccharide units. The ^{13}C NMR spectra of both the negatively charged EPSs (EPS 1−2 and 2−2) were similar, and they were also similar to the negatively charged EPS from using glucose carbon source (unpublished results), suggesting mannose monomers with an α-(1,3)-type glycosidic bond. All the chemical shifts of ^{1}H and ^{13}C NMR were analyzed by the CASPER (Computer Assisted Spectrum Evaluation of Regular Polysaccharides) web server, and the simulation showed low deviation from the putative results.[45] All four EPSs were also tentatively considered as having a linear structure due to the limited number of peaks from the ^{13}C NMR spectra.

Thermal Stability and XRD Analysis. Thermogravimetric analysis was employed to study the thermal stability of all the purified EPSs (Figure S1 in the SI). The degradation temperature (T_d) of neutral EPS 1−1 and 2−1 was 203 and 227 °C, respectively, which were both much lower than that of the neutral EPS when using glucose as carbon source (319 °C) (unpublished results). Meanwhile, the negatively charged EPSs had a T_d of 316 °C (EPS 1−2) and 309 °C (EPS 2−2), similar to that of the negatively charged EPS from the glucose substrate (unpublished results). However, both EPS 1−2 and 2−2 had a minor weight loss component in the temperature range of 200 to 250 °C, which may arise from impurities in these EPSs.

In XRD analysis, the crystalline fraction gives sharp narrow peaks while the amorphous phase exhibits a very broad peak.[46] The glucomannan-type EPS 1−1 and 2−1 both showed strong and broad amorphous peaks in their diffractograms and had a crystallinity index of 0.02 and 0.01, respectively, indicating the highly amorphous nature of these EPSs. Both of the mannan-type EPSs, 1−2 and 2−2, exhibited an intense and narrow diffraction peak at 31.6° with an inter planar spacing (*d*-spacing) of 0.283 nm, and a crystallinity index of 0.17 and 0.14, respectively (Figure 6). These TGA and XRD results suggest that there may be some direct or indirect connection between the minimal degree of crystallinity of EPS 1−1 and 2−1 and their relatively low thermostability. The XRD profiles may also reflect the essential difference of monomer composition between the glucomannan and mannan type EPSs.

Cytotoxicity of EPSs on HEK-293 Cells. To assess the biocompability of the EPSs produced by strain *Geobacillus* sp. WSUCF1 using corn stover as substrate, a cytotoxicity assay was performed with HEK-293 cell line. The HEK-293 cell line is a robust cell culture model for cytotoxicity assay in biopharmaceutical research, and it has been well characterized for its relevance to the toxicity models in human.[47−49] The four EPSs were tested at a wide array of concentrations (500 to 10 000 μg/mL). The cell viability of HEK-293 cells remained unaffected by all four EPSs as a function of dose (Figure 7). For the EPSs isolated at 24 h, EPS 1−1, the highest noncytotoxic concentration was 3000 μg/mL, while EPS 1−2 had a highest noncytotoxic concentration of 4000 μg/mL. While we are aware of noncytotoxic concentration values against HEK-293 cells as high as 1000 μg/mL for EPS from mesophilic bacteria,[50] the values obtained for EPS 1−1 and 1−2 in the present study indicate that these thermophilic EPSs exhibit higher nontoxic and biocompatibility properties to human embryonic kidney cells. Furthermore, both EPS 1−1 and 1−2 from corn stover demonstrated higher noncytotoxic concentration compared with the corresponding EPSs obtained when using glucose as carbon source, whereas EPS 2−1 and 2−2 showed similar noncytotoxic concentrations to the EPSs from glucose medium (unpublished results). The enhanced noncytotoxicity of the EPSs from using corn stover as substrate indicates that the unprocessed lignocellulosic agricultural waste could be a promising resource for the production of EPSs with outstanding biocompatibility properties using thermophiles with lignocellulolytic capability.

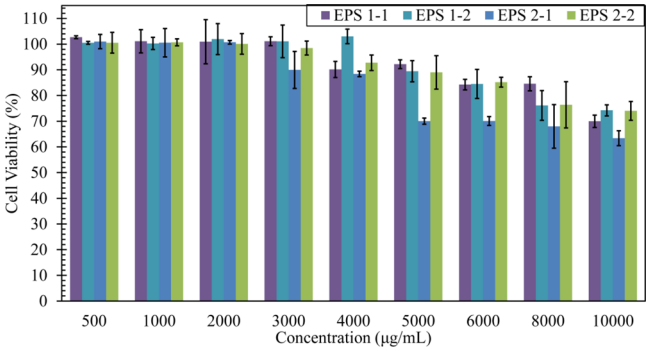

Figure 7. Effect of EPSs produced by *Geobacillus* sp. WSUCF1 using corn stover on HEK-293 cells.

Antioxidant Properties. The antioxidant activities of the four EPSs from strain WSUCF1 in vitro were evaluated using biochemical methods against hydroxyl, DPPH, and superoxide anion radicals. For all 4 EPSs, the highest scavenging activities against hydroxyl radicals were in the range of 16% to 19% (Figure 8a). EPS 1−1 and 1−2 had moderate scavenging activities (around 30%) against DPPH radicals at a high dose of 10 mg/mL (Figure 8b). Meanwhile, both of the neutral EPSs showed relatively high antioxidant activities against superoxide anion radicals: at the concentration of 10 mg/mL, EPS 1−1 had the maximum scavenging activity of 51% against these radicals (Figure 8c). Natural polysaccharides with antioxidant activity are of special interest since they can be applied to replace synthetic antioxidants, which have undesirable side effects, for the prevention of neuronal degeneration. The disclosure of structure−function relationships of antioxidant exopolysaccharides in future research will provide direction for further structural modification to enhance their antioxidant capabilities.

Green Metrics Calculations. The green metric of EPS production via thermophilic fermentative synthesis was calculated for using two different carbon sources (glucose and unprocessed corn stover). Assuming the EPS and bacterial cell mass are the products, the *E*-factor of the corn stover process is lower than that of using glucose (Table 3). Meanwhile, the EPS producing process using corn stover has a higher product-to-input ratio compared with using glucose, indicating the material efficiency of thermophilic EPS production when using corn stover is relatively higher that of its production from glucose. For the energy efficiency, the calculations have been made employing the energy density of the nutrients, products as well as bacterial cell mass. The second generation of lignocellulosic biomass feedstock was considered with lower quality in sugar equivalency.[24] However, EPS production from unprocessed corn stover shows a better energy efficiency due to the coproduction of more bacterial cell mass which contributes positively to the energy of products.

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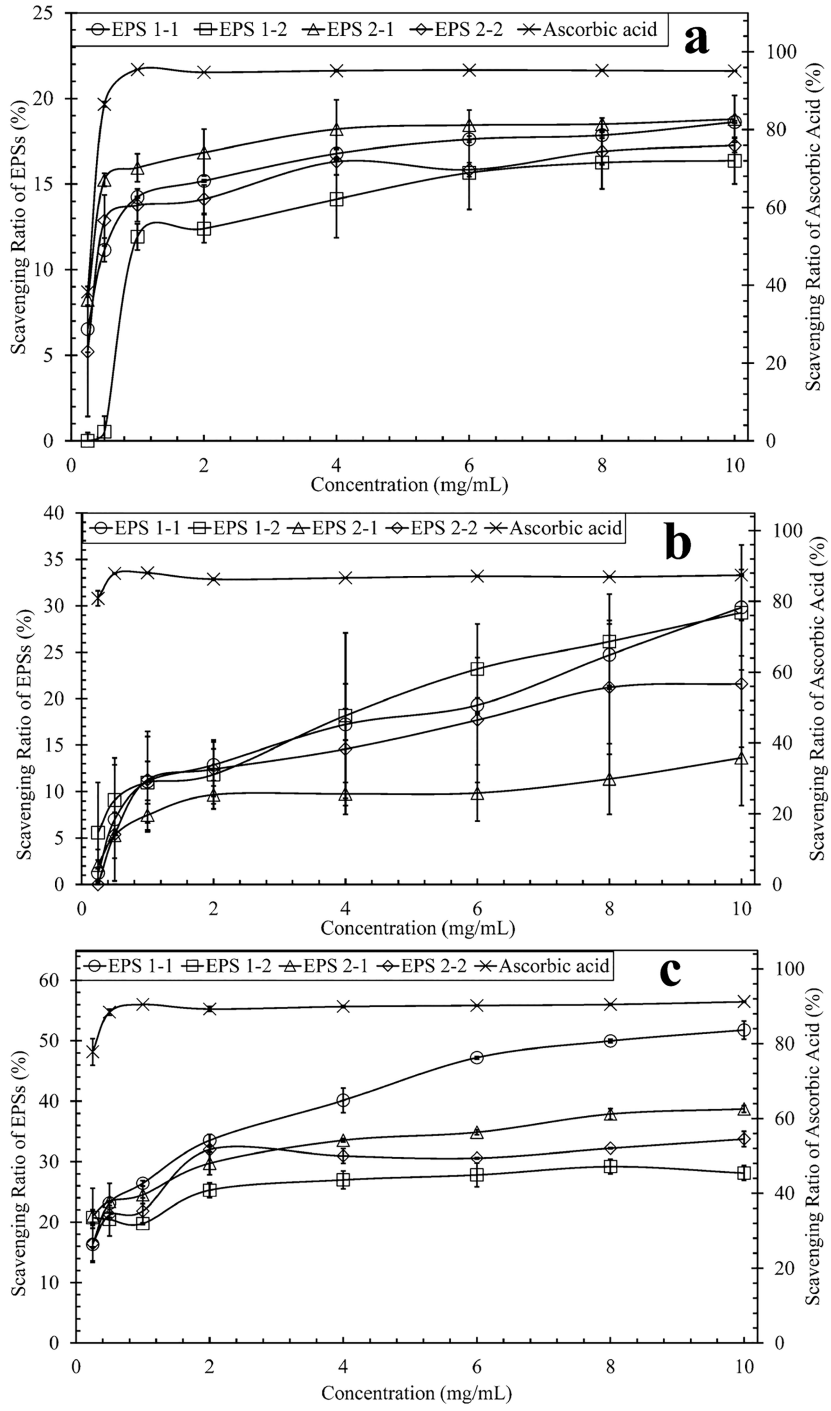

Figure 8. Free radical scavenging capabilities of EPSs from *Geobacillus* sp. WSUCF1 using corn stover as substrate, (a) $OH^{\bullet}$ scavenging activity, (b) $DPPH^{\bullet}$ scavenging activity, and (c) $O^{2-\bullet}$ scavenging activity. Values are means with standard error bars, and error bars smaller than the symbols are not shown.

Table 3. Comparison of the Sustainability Metrics for the EPS Production Using Glucose and Unprocessed Corn Stover

process	*E*-factor	product-to-input ratio (%)	energy efficiency (%)	land use (ha/kg)	raw material cost (USD/kg)
glucose	774	0.129	9.75	0.00129	91.3
corn stover	731	0.137	12.49	0.00146	83.7

The land use per EPS unit mass was calculated from the average corn crop yields for glucose and lignocellulosic materials. The land use per unit mass of lignocellulose-derived EPS production was higher than that of using glucose. The land use is usually a function of land productivity and crop composition.[24] However, the glucose is obtained from the hydrolyzation of starch isolated from corn crop, which is in direct competition with arable land resources for food.[51] The

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successful utilization of agricultural lignocellulosic waste for EPS production will provide a significant reduction in arable land use, and thus more farmland can be applied for the production of edible crops. Finally, the raw material costs were calculated per kilogram of EPS produced (Table 3). The economics of the fermentative process is an important factor to determine its feasibility and implementation, and the economical raw material can lead to significantly lower financial investment, making the process more attractive.[52,53] The relatively lower substrate cost when using unprocessed corn stover for EPS production can be the driving force for further studies concerning the enhancement of fermentation process. However, the removal of the pretreatment process of corn stover also considerably reduces the production cost of EPSs. The fermentable sugar production cost was considered as $0.42/kg using dilute sulfuric acid for pretreatment, and $1.41/kg by the enzymatic method.[54] For the production process of EPS using unprocessed corn stover, maximumly $20.63 can be reduced for the production cost of 1 kg of EPS based on the estimation of pretreatment costs.

CONCLUSIONS

A thermophilic bacterium *Geobacillus* sp. WSUCF1, which has both EPS producing and lignocellulolytic capabilities, produced 410 mg/L of EPS with a specific yield of 0.53 g EPS/g cell dry weight in a 40-L bioreactor using unprocessed corn stover as carbon source. Four EPSs were obtained after the purification process. Both the neutral EPSs had a linear glucomannan-type structure, mainly composed of α-(1,3)-linked mannose and α-(1,6)-linked glucose, while the negatively charged EPSs were a linear mannan-type structure with α-(1,3)-linked mannoses. The thermostability of both neutral EPSs was lower than the neutral EPS when using glucose as carbon source, but the biocompatibility of the EPSs produced at 24 h were exceptionally high, with enhanced noncytotoxicity compared with the EPSs produced using a glucose medium. The neutral EPS produced at 24 h also showed potential as antioxidant reagents against superoxide anion radicals. The concise green metrics demonstrated that the use of unprocessed corn stover in EPS production can compete successfully with using traditional pure sugar substrate. With both biocompatible and antioxidant properties, the EPSs produced by thermophilic bacterium *Geobacillus* sp. WSUCF1 using corn stover provides a wide range of potential applications in the biomedical, food, and cosmetic industries.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssuschemeng.0c02435.

Figure S1, TGA and DTG graphics of EPSs; Table S1, methods for green metrics calculations, substrate consumption per kilogram EPS using glucose; Table S2, substrate consumption per kilogram EPS using corn stover; Table S3, energy per kilogram EPS using glucose; Table S4, energy per kilogram EPS using corn stover; Table S5, land use of glucose and corn stover; and Table S6, inventory of raw material cost (PDF)

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Notes

The authors declare no competing financial interest.

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