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# Enhancing digestibility of *Miscanthus* using lignocellulolytic enzyme produced by *Bacillus*

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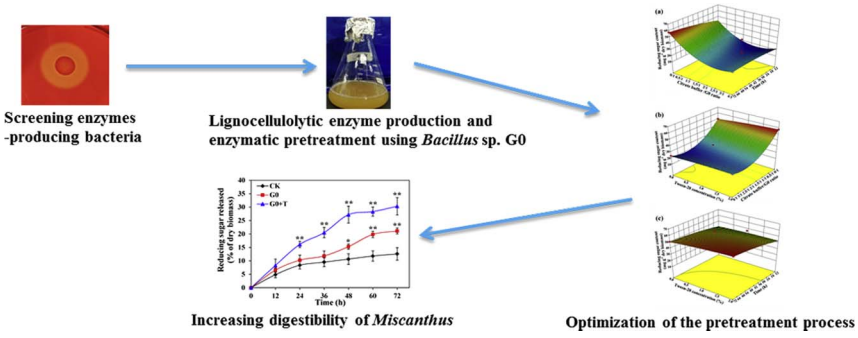
## GRAPHICAL ABSTRACT




## ARTICLE INFO

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## ABSTRACT

In this study an effective bacterial pretreatment method was developed to improve digestibility of *Miscanthus*. Seven new bacterial isolates, which showed excellent xylanase production ability using *Miscanthus* as carbon source, were used to perform the pretreatment experiments. After pretreatment, the hemicellulose content and crystallinity index of *Miscanthus* were decreased, while the reducing sugars released from *Miscanthus* were significantly increased by 30.8–87.8% after enzymatic hydrolysis. *Bacillus* sp. G0 was selected to optimize the pretreatment parameters via response surface methodology due to its high reducing sugars released from *Miscanthus*. According to the optimal model, the pretreatment parameters were set as citrate buffer/G0 fermentation broth ratio at 0.34, pretreatment time at 100 h and Tween-20 concentration at 1.73%. The reducing sugars released from *Miscanthus* pretreated by optimal parameters were 305 mg $g^{-1}$ dry biomass. The results suggested our bacterial pretreatment approaches have great potential to increase digestibility of bioenergy crops.

## 1. Introduction

In order to decrease dependence on traditional fuels and reduce related environmental and economic threats, biofuels from renewable lignocellulosic feedstocks have received increasing interest as an alternative to fossil fuels. Lignocellulosic biomass is composed of 55–75% carbohydrates (cellulose and hemicellulose) by dry weight, which can be used to produce ethanol and other substitutable chemicals (Binder

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and Raines, 2009). However, the specific structure of cellulose, tightly packed by glucose polymer chains, makes cellulose water insoluble and tolerant to decomposition (Keiluweit et al., 2010). Another main plant cell wall polysaccharide, hemicellulose, which is a diverse group of short-chain branched and usually disorderly twined with cellulose microfibrils networks. The most abundant hemicellulose is xylan polymer, which possesses a great variety of linkage and branching types and forms an overlying layer via hydrogen and covalent bonds with cellulose and lignin, respectively (Dufresne, 2008). The complex hemicellulose structures cause a physical barrier that limits the degradation of cellulose by microbes and enzymes (Moreira, 2016). In addition, lignin, the most abundant aromatic polymer, forms an insoluble, unreactive layer around the carbohydrates through ester and ether bonds, thus further preventing the cellulose from breaking down (Studer et al., 2011).

To weaken biomass recalcitrance, two strategies have been raised to improve the access of enzymes to cellulose via either removal of hemicellulose or delignification of biomass (Kalluri et al., 2014; Sindhu et al., 2016). The delignification of biomass has been widely studied to bare the polysaccharide composition by various physical, chemical and biological pretreatment processes during the last few decades (Kumar et al., 2013; Singh et al., 2014). However, it has been reported that the hydrolyzability of cellulose is mainly determined by cellulose crystallinity, which is negatively correlated with biomass digestibility (Li et al., 2013; Xu et al., 2012). Hemicellulose has been proved to dramatically influence cellulose crystallinity due to its polymer chains embedding the crystalline cellulose elementary fibrils (Li et al., 2013; Moreira, 2016). Cellulose hydrolysis by cellulase is influenced more by the removal of xylan than lignin because xylan removal directly affects glucan chain accessibility (Sindhu et al., 2016). The removal of xylan can also facilitate to decrease enzyme inhibition by xylo-oligomers and the requirements of accessory enzymes (Xue et al., 2015). Moreover, unlike delignification, the removal of hemicellulose produces fewer inhibitors, which will inhibit the next stages of enzymatic hydrolysis and yeast fermentation (Mosier et al., 2005).

Biological pretreatment using microorganism in nature has been proposed as an economical, eco-friendly technology to improve biomass digestibility (Sindhu et al., 2016). Lignocellulolytic enzymes produced by microorganisms, including carboxymethyl cellulase (CMCase), xylanase, phenol oxidase, chitinase, peroxidase, cellobiohydrolase and laccase etc., play an important role in rapid lignocellulose degradation (Woo et al., 2014). Fungi usually grow slowly and require a long time to produce lignocellulolytic enzymes, thus causing more cellulose and hemicellulose loss in the pretreatment process (Shrestha et al., 2015; Vasco-Correa et al., 2016). Most bacteria can keep comparatively rapid growth in ordinary media and utilize a very wide range of different substances as nutrient sources to produce various enzymes in few hours (Glick, 2014).

*Miscanthus* is one of the most important bioenergy crops for bioethanol production in the world due to its high photosynthesis efficiency, high biomass yield and remarkable adaptability to various environmental conditions (McCalmont et al., 2017). To break the recalcitrant structure of *Miscanthus*, various physical, chemical and fungal pretreatment methods have been reported (Boonmanumsin et al., 2012; Serrano et al., 2010). However, little is known about the effects of bacterial pretreatment on the digestibility of *Miscanthus*. In the current study, a total of seven lignocellulolytic enzyme-producing bacteria isolated from forest soils and paper mill sludge were selected to produce lignocellulolytic enzymes using *Miscanthus* biomass as carbon source in a short time. Then the fermentation broths were continually cultured in the optimal temperature and pH of xylanase to remove hemicellulose of *Miscanthus*. The pretreatment process was further optimized by *Bacillus* sp. G0 via response surface methodology (RSM). In addition, the biomass digestibility of bacterial pretreated *Misccathus* was also evaluated by enzymatic hydrolysis.

## 2. Materials and methods

### 2.1. Materials

The mature stem tissues of *Miscanthus* collected from the Daming Mountain Scenic Area in Linan, Zhejinag Province of China, in October 2015, were dried at 50 °C until constant weight. Then the dried biomass was ground through a 50 mesh sieve and stored in a dry glass container until use. Carboxymethyl cellulose (CMC), beechwood xylan and 3,5-dinitrosalicylic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Filter paper (Whatman No.1) was obtained from ThermoFisher Scientific (San Jose, CA, USA). Cellulase (Celluclast 1.5 L) from *Trichoderma reesei* and beta-glucosidase (Novozyme 188) from *Aspergillus niger* were obtained from Novozymes (Novozymes, Franklington, USA). All other chemicals and reagents were of analytical grade.

### 2.2. Isolation and identification of lignocellulolytic enzyme-producing bacteria

The bacteria used in this study were isolated from forest soil or paper mill sludge (Thunder Bay, Ontario, Canada). Briefly, about 5.0 g of samples were suspended in 50 mL of sterile distilled water; the mixture was shaken at 200 rpm and 37 °C for 30 min. Then, the isolate was spread via the standard serial dilution plate method using a Luria-Bertani (LB) medium. After incubating at 37 °C for 1–2 days, colonies of bacteria that showed different morphological features, like size and color, were cultured in LB medium for 24 h. Then 5 μL of each pure culture was dropped on a CMC and a xylan agar plate, which consisted of mineral salt medium (0.1% $NaNO_3$, 0.1% $K_2HPO_4$, 0.1% KCl, 0.05% $MgSO_4$, 0.05% yeast and 0.3% peptone), 0.5% (w/v) CMC or xylan and 1.5% agar. All of the plates were incubated at 37 °C for 2 days and stained with 0.1% (w/v) Congo red solution for 30 min and visualized by washing with 1 M sodium chloride solution for 10 min. Then the hydrolysis ability was calculated according to the method of Guo et al. (2017). The strains simultaneously showing higher CMC and xylan hydrolysis abilities were used for pretreatment experiment. For identification of these bacteria, their genomes were extracted with a Bacteria DNA kit (Bio Basic, Markham, Ontario, Canada). The internal transcribed spacer (ITS) region was amplified using universal primers HAD-1 (5′-GACTCCTACGGGAGGCAGCAT-3′) and E1115R (5′-AGGGTTG CGCTCGTTGCGGG-3′) and purified for sequencing. Then these sequences were analyzed by BLAST and submitted to NCBI (https://www.ncbi.nlm.nih.gov/).

### 2.3. Inoculum preparation and pretreatment

#### 2.3.1. Screening the best Miscanthus-degrading bacteria

The screened seven CMC/xylan-degrading bacteria were firstly cultured in the above mineral salt medium containing 0.5% (w/v) *Miscanthus* biomass to produce various lignocellulolytic enzymes at 37 °C, with agitation at 200 rpm. After 6 h of incubation, all bacteria obtained a maximum xylanase activity with the values of 182.3–208.1 U $g^{-1}$ dry biomass, while the FPase and CMCase activities ranged from 0.2 to 1.0 U $g^{-1}$ dry biomass and 4.8–21.1 U $g^{-1}$ dry biomass, respectively. To achieve maximum degradation of hemicellulose, after 6 h of incubation the pH of bacterial fermentation broth was adjusted to 5.5 using 2 M hydrochloric acid (HCl) and then brought to 2-fold volumes of fermentation broth using 50 mM citrate buffer (pH 5.5). Then, the mixture was incubated at 55 °C for 72 h with agitation at 150 rpm. The culture solution incubated without bacteria was used as the control. The supernatant was collected by centrifugation at 12,000*g* for 3 min after 6, 12, 24, 36, 48, 60 and 72 h of incubation and used to determine the reducing sugar content. Tetracycline (40 mg $L^{-1}$) and sodium azide (0.005%, w/v) were used to control the growth of the bacteria. To measure whether the bacteria could still grow during the

pretreatment process, the mixed cultures of these bacteria, after 6, 24, 48 and 72 h of incubation, was spread on a LB agar plate and cultured at 37 and 55 °C. After 72 h of incubation, the samples were washed with distilled water to remove the bacterial cells through a double-layered muslin cloth with the meshes of 300. Then solid residues were oven dried at 105 °C until constant weight and used for cell wall composition analysis, X-ray powder diffraction (XRD) analysis and subsequent enzymatic hydrolysis. The biomass weight loss (%) was calculated as the percentage of the loss dry weight after pretreatment and initial dry weight of sample.

#### 2.3.2. Optimization of the pretreatment parameters using Bacillus sp. G0

For optimization of the pretreatment experiments, only the strain *Bacillus* sp. G0 was used due to its high release of reducing sugars and low cellulose crystallinity index (CrI). To obtain an enzymatic solution, the lignocellulolytic enzymes produced by *Bacillus* sp. G0 using 0.5, 1, 2, and 4% *Miscanthus* biomass as the sole carbon source were monitored after 6, 12, 24, 36, 48, 60 and 72 h of incubation. The effects of pH on the activities of FPase, CMCase and xylanase were determined in a pH range of 4.0–7.0. The optimal temperature was determined over the temperature range of 40–80 °C. To evaluate the effects of pretreatment time, G0 fermentation broth/ citrate buffer ratio and surfactant Tween-20 concentration on the reducing sugar production in the pretreatment process, an experimental design matrix was formulated by using Design-Expert software (Version 8.0.6., Stat-Ease Inc., Minneapolis, USA). The matrix consisted of 20 experiments and all experiments were performed at 60 °C, pH 5.5 with agitation at 150 rpm. The *Bacillus* sp. G0 was cultured using 0.5% *Miscanthus* as carbon source to produce various lignocellulolytic enzymes. After 6 h of incubation (with the highest xylanase activity), G0 fermentation broth was mixed with different volumes of citrate buffer. The pH was first adjusted to 5.5 using 2 M HCl and then brought to different volumes using 50 mM citrate buffer (pH 5.5).

### 2.4. Biomass cell wall composition analysis

The cell wall composition was measured according to the description of Ibáñez and Bauer (2014) and Shrestha et al. (2015) with some minor modifications. Briefly, about 0.1 g pretreated and untreated dry biomasses were extracted four times at 65 °C for 30 min each: twice in 1.5 mL hot water, once in 1.5 mL absolute ethanol and once in 1.5 mL acetone. The extractive-free residue was air-dried in a chemical hood for 2 days and weighted to calculate the content of extractives. Then the dry samples were incubated with 1 mL of 72% sulfuric acid at 30 °C with vortexing every 15 min. After 1 h, the hydrolysate was diluted to 3% sulfuric acid by the addition of deionized water and autoclaved at 121 °C for 1 h before storage at 4 °C overnight to settle the solids. Finally, the supernatant and solid residues were separated by centrifugation at 12,000*g* for 3 min. The supernatant was carefully removed and used to determine the content of hexose and pentose using the anthrone-sulfuric acid method and orcinol-hydrochloric acid method, respectively. The solid residues were used to measure the content of Klason lignin according to the method of Ibáñez and Bauer (2014).

### 2.5. Enzymatic hydrolysis

Enzymatic hydrolysis was performed according to NREL laboratory analytical procedure LAP 009. The solid residues of pretreated and untreated *Miscanthus* biomass were loaded at a solid concentration of 2.0% (w/v) in a 50 mM citrate buffer (pH 4.8) containing 0.005% (w/v) sodium azide. Cellulase (Celluclast 1.5 L) and beta-glucosidase (Novozyme 188) were used for hydrolysis experiments. The enzyme loadings of Celluclast 1.5 L and Novozyme 188 were 20 FPU $g^{-1}$ glucan and 30 CBU $g^{-1}$ glucan, respectively. The hydrolysis experiment was conducted at 50 °C and 150 rpm in a shaking incubator for 72 h. After enzymatic hydrolysis, the mixture was centrifuged at 12,000*g* for 3 min and the supernatants were collected for measurement of reducing sugar content. The reducing sugars released were calculated as a percentage of the dry weight of biomass.

### 2.6. Determination of enzyme activates and reducing sugars

The filter paper activity (FPase), CMCase and xylanase activities were measured using filter paper, carboxymethyl cellulose and xylan as substrate, respectively, according to the description of Guo et al. (2017). The reducing sugars were determined using the 3,5-dinitrosalicylic acid (DNS) reagent.

### 2.7. XRD, FTIR and SEM analysis

X-ray diffraction patterns were collected using a PANanalytical X'pert Pro diffractometer (PANalytical, Holland) equipped with a conventional X-ray tube ($CuK_\alpha$ 40 kV, 20 mA, line focus) in transmission mode. The patterns of all samples were recorded in the range of 10–45° (2θ) at a step size of 0.0263° and 165 s/step. The CrI was calculated using the intensity of crystalline portion ($I_{002}$, θ = 22.5°) and the intensity of amorphous portion ($I_{am}$, θ = 18.5°) as follows according to the method of Segal et al. (1959): $\text{CrI}\ (\%) = \frac{I_{002} - I_{am}}{I_{002}} \times 100$.

Fourier transform infrared spectroscopy (FTIR) analysis was performed by a Bruker Tensor 37 FTIR Spectrophotometer (Bruker Optics, Inc., Billerica, MA). The spectra of oven-dried samples were recorded in transmission mode with 32 scans at a spectral resolution of 4 $cm^{-1}$ within the 4000–600 $cm^{-1}$ range.

For scanning electron microscope (SEM) observation, the oven-dried samples were sputter-coated with gold and observed by scanning electron microscope (SEM Hitachi AU-70, Tokyo, Japan).

### 2.8. Statistical analysis

All the experiments were performed in triplicates or quadruplicate, and the results were shown as mean ± SD. Statistical analysis was carried out by one-way analysis of variance using SPSS (SPSS Inc., USA, version 13.0).

## 3. Results and discussion

### 3.1. Screening and identification the lignocellulolytic enzyme-producing bacteria

In total 21 different bacterial strains were isolated according to the bacterial size and color, including 9 strains from forest soil and 12 strains from paper mill sludge. After staining using Congo red, 4 strains from forest soil (G0, GA1B, GS1 and H1) and 3 strains from paper mill sludge (P1, P2 and P3), simultaneously showing large halo in CMC and xylan plates, were selected to perform the next experiments (Fig. 1). Strains G0, GA1B, H1, P2 and P3 were identified as *Bacillus* sp., GS1 as *Pseudomonas* sp. and P1 as *Raoultella* sp. according to the analysis of 16S rRNA sequences, which have been deposited in the NCBI GenBank and obtained the accession numbers (Table 1). Congo red staining was used for preliminary screening of the cellulase active bacterial and fungal species, and the size of halo diameter was positively related to the activity of cellulase (Colonia and Junior, 2014; Woo et al., 2014). Kamrun et al. (2015) used Congo red staining method to screen a cellulolytic fungus, which showed excellent CMCase and FPase activities and a great potential for the degradation of organic solid waste. Two bacterial strains *Cellulosimicrobium cellulans* L804 and *Bacillus agaradhaerens* C9 showed the clearing halos in the CMC or xylan containing agar plate with Congo red staining were able to secret CMCase and xylanase by degrading lignicellulosic biomass (Liu et al., 2017). The hydrolysis abilities of these bacterial colonies were calculated as the

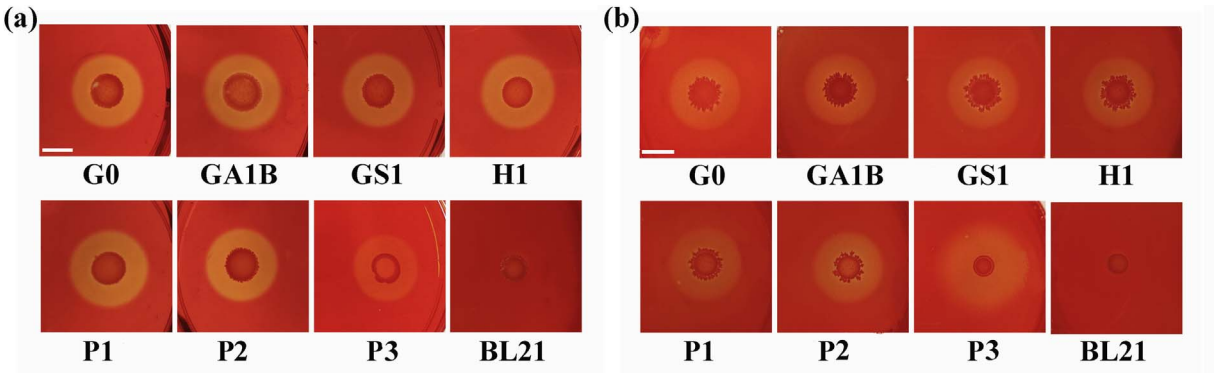


**Fig. 1.** Evaluating the degradation ability of biomass-degrading bacteria to CMC (a) and xylan (b) using CMC and xylan as carbon source, respectively, by staining with 1% Congo red solution. Negative: *E. coli* BL21. Bar = 1 cm.

square of the halo diameter/colony diameter ratio to further verify the CMCase and xylanase production ability of these bacteria (Table 1). Eight bacterial strains with high hydrolysis activity values were previously reported to produce multiple lignocellulolytic enzymes (CMCase, FPase, xylanase and laccase) and hydrolyze the cell wall of microalgae (Guo et al., 2017). *Bacillus* sp. 65S3 and *Pseudomonas* sp. CDS3 showed better hydrolysis activity of CMC using Gram's iodine staining were able to produce bioethanol and xylitol using agave biomass as substrate (Xiong et al., 2014).

### 3.2. Positive effects of bacterial pretreatment on Miscanthus digestibility

The reducing sugar contents were continuously measured to evaluate the degradation ability of these bacteria in the process of pretreatment. The reducing sugar contents of all bacteria were significantly enhanced with increased pretreatment time, compared to that of control (Fig. 2). After 72 h of pretreatment, the reducing sugar content of strains G0, GA1B, GS1, H1, P1, P2 and P3 were increased by 84.3, 87.0, 71.4, 77.5, 88.8, 81.2 and 68.1%, respectively, compared to that of control (Fig. 2). The most biomass loss was found after pretreatment of strain GA1B with a percentage of 13.0%, while the strain G0 showed relatively small biomass loss, with the highest solid recovery of 94.5%. For the other five strains, their dry weight loss ranged from 7.5% to 10.5% after bacterial pretreatment (Table 2). This is similar with that the biomass weight losses were significantly different after incubating different fungi species with *Miscanthus* biomass (Shrestha et al., 2015). The biomass weight loss after pretreatment with microorganisms was closely related to the kinds and concentrations of lignocellulolytic enzymes produced by fungi and bacteria (Liu et al., 2015; Oke et al., 2017).

The cell wall compositions significantly differed after different bacterial pretreatments (Table 2). All bacterial pretreatments reduced the contents of extractive and hemicellulose, and increased the content of the cellulose, while there was almost no change in Klason lignin content (Table 2). The greatest hemicellulose loss was caused by pretreatment with strain G0 and GA1B, which decreased by 23.1 and 24.9%, respectively, compared to that of the untreated control (Table 2). The decrease of hemicellulose after pretreatment may be mainly due to their higher activities of xylanase than FPase and CMCase. In addition, the pretreatment conditions, including temperature and pH, were selected according to the optimal temperature and pH of xylanase. The increased cellulose content may be partially attributed to the reduction of hemicellulose and thus making more cellulose available (Öhgren et al., 2007). To confirm whether bacterial pretreatment influenced the cellulose crystalline structures of *Miscanthus* biomass, the CrI of pretreated and untreated biomass was determined by a XRD analysis. The CrI of untreated control was 44.5%, which was similar with that the CrI was ranged from 33.84 to 55.9% for various untreated *Miscanthus* species (Xu et al., 2012). However, after pretreatment the CrI was 27.5, 41.4, 39.9, 30.1, 30.5, 41.7 and 44.6% for the strains G0, GA1B, GS1, H1, P1, P2 and P3, respectively (Table 2). The XRD spectrum analysis showed that in the pattern of the control *Miscanthus* appeared a sharp high peak at 2θ 22.5° (002 lattice plane) and a broad hump at 2θ 15.5° (101 lattice plane), which represents the typical cellulose I structure. These peaks had different degrees of reduction, especially after the pretreatment of strains G0, H1 and P1. This further confirmed that bacterial pretreatment may degrade the folding layer of cellulose to disrupt the crystal structures (Wu et al., 2010). The reducing sugars released from all bacterial pretreatment biomass was significantly enhanced after enzymatic saccharification, especially from G0 pretreatment biomass with up to 87.8% increase in sugar production, compared to that from untreated biomass (Fig. 3). This was consistent with that the degradation of hemicellulose can positively affect lignocellulose enzymatic digestibility (Li et al., 2013) and the reduction of cellulose CrI resulted in efficient cellulase enzyme penetration and high affinity to cellulose substrate (Saha, 2003; Xu et al., 2012).

**Table 1**
CMC and Xylan hydrolytic abilities of seven different bacteria and one negative control. Values represent mean ± SDs (n = 3).

| Isolates | Genus | Accession No. | CMC | | | Xylan | | |
|---|---|---|---|---|---|---|---|---|
| | | | HD (D, cm) | CD (d, cm) | HA $(D/d)^2$ | HD (D, cm) | CD (d, cm) | HA $(D/d)^2$ |
| G0 | *Bacillus* | MF462251 | 2.46 ± 0.05 | 1.01 ± 0.07 | 5.93 | 2.48 ± 0.09 | 0.94 ± 0.06 | 6.96 |
| GA1B | *Bacillus* | MF462252 | 2.59 ± 0.11 | 1.09 ± 0.09 | 5.59 | 2.52 ± 0.10 | 0.99 ± 0.02 | 6.43 |
| GS1 | *Pseudomonas* | MF462253 | 2.52 ± 0.08 | 1.00 ± 0.03 | 6.37 | 2.53 ± 0.09 | 1.05 ± 0.07 | 5.79 |
| H1 | *Bacillus* | MF462254 | 2.42 ± 0.05 | 1.00 ± 0.12 | 5.87 | 2.45 ± 0.10 | 1.02 ± 0.05 | 5.8 |
| P1 | *Raoultella* | MF462255 | 2.51 ± 0.11 | 1.12 ± 0.07 | 5.00 | 2.49 ± 0.29 | 1.00 ± 0.11 | 6.2 |
| P2 | *Bacillus* | MF462256 | 2.48 ± 0.12 | 1.05 ± 0.08 | 5.54 | 2.50 ± 0.12 | 0.95 ± 0.14 | 6.86 |
| P3 | *Bacillus* | MF462257 | 2.37 ± 0.11 | 1.01 ± 0.01 | 5.54 | 2.38 ± 0.11 | 0.83 ± 0.02 | 8.21 |
| Negative | *E. coli* BL21 | – | – | 0.90 ± 0.05 | – | – | 0.71 ± 0.04 | – |

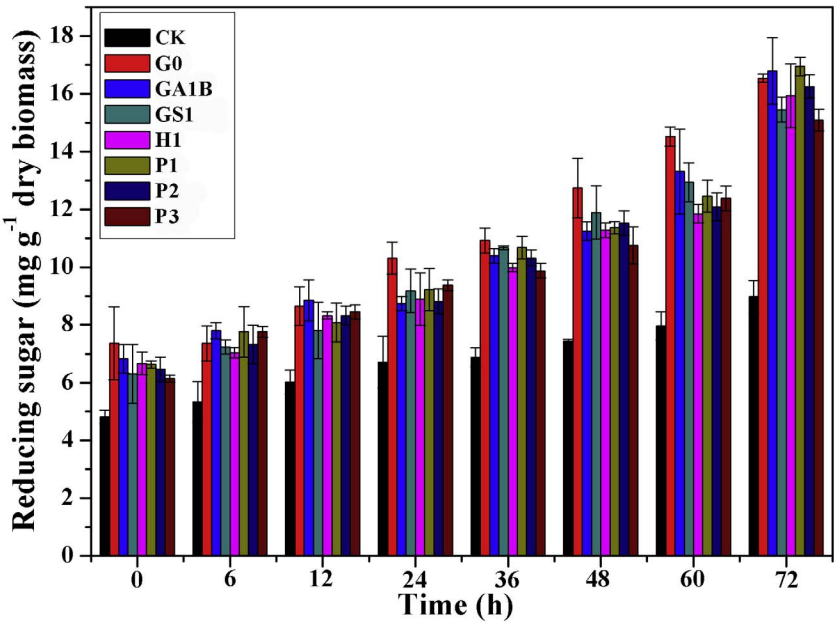


**Fig. 2.** Yields of reducing sugars from *Miscanthus* by different bacteria pretreatment. The crude enzymes produced using 0.5% *Miscanthus* biomass as carbon source were mixed with citrate buffer in a ratio of 1:1 (v/v). Values represent mean ± SDs (n = 4).

### 3.3. Optimization of the pretreatment process parameters using Bacillus sp. G0

#### 3.3.1. The enzymatic characteristics of Bacillus sp. G0

Four different substrate concentrations were used to evaluate the production of lignocellulolytic enzymes using untreated *Miscanthus*. 0.5% *Miscanthus* biomass induced the highest FPase, CMCase and xylanase activities with the values of 2.2, 11.8 and 191.4 IU $g^{-1}$ dry biomass, after 24, 12 and 6 h of incubation, respectively, while correspondingly maximum of them were only 0.6, 2.2 and 25.1 IU $g^{-1}$ dry biomass when 4% substrate concentration was used. It has been reported that lignocellulolytic enzymes produced by fungi and bacteria were mainly affected by the carbon and nitrogen sources in the medium (Dashtban et al., 2011; Mikiashvili et al., 2006). The activity of extracellular hydrolytic and oxidative enzymes were increased by gradually increasing the concentration of lignocellulosic substrates in *Pleurotus dryinus* IBB 903, but their accumulation was decreased by further increasing of the substrate concentration due to higher viscosity of medium and decreased mass exchange and aeration (Elisashvili et al., 2006). The optimal pH values for FPase, CMCase and xylanase were all 5.5. The highest FPase and CMC activities were found at 55 °C, while the optimal temperature of xylanase was 60 °C. These were similar to most bacteria with optimal pH ranging from 4.5 to 7.0 and optimal temperature ranging from 37 to 65 °C (Beg et al., 2001; Sadhu and Maiti, 2013).

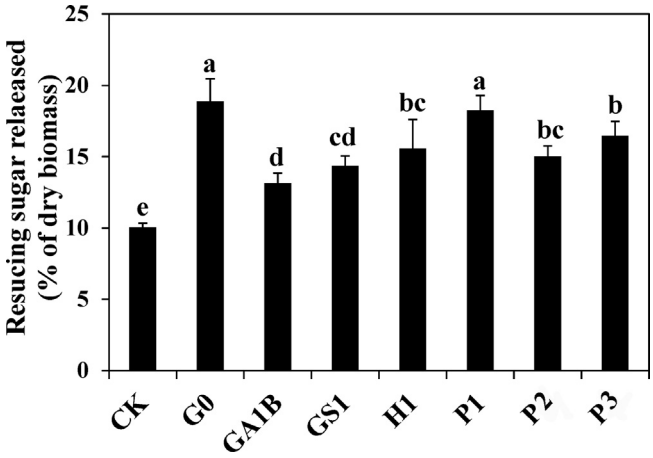


**Fig. 3.** Reducing sugars released from bacterial pretreated *Miscanthus* after enzymatic hydrolysis. Values represent mean ± SDs (n = 4). Different letters indicate significant differences ($p < 0.05$) among different bacteria.

#### 3.3.2. Optimal experiments via response surface methodology

The reducing sugar production during pretreatment process was used as the optimal response parameter due to its positive correlation to reducing sugars released after enzymatic hydrolysis ($R^2 = 0.7809$, data

**Table 2**
Biomass weight loss, cell wall composition and cellulose crystallinity of *Miscanthus* after pretreatment of seven different bacteria. Values represent mean ± SDs (n = 3).

| Pretreated | Biomass wt. loss (%) | Composition (%) | | | | CrI (%) |
|---|---|---|---|---|---|---|
| | | Extractive | Glucan | Xylan | Klason lignin | |
| Untreated | – | 8.32 ± 0.99 | 36.66 ± 2.85 | 32.10 ± 2.97 | 25.2 ± 2.83 | 44.5 |
| G0 | 5.5 | 5.37 ± 1.11 | 42.18 ± 0.98 | 24.70 ± 2.55 | 24.3 ± 0.71 | 27.5 |
| GA1B | 13.0 | 5.51 ± 1.32 | 38.01 ± 4.11 | 24.12 ± 1.94 | 25.6 ± 1.27 | 41.4 |
| GS1 | 10.5 | 6.29 ± 0.85 | 45.55 ± 3.00 | 27.53 ± 1.45 | 26.0 ± 2.28 | 39.9 |
| H1 | 9.5 | 4.74 ± 0.21 | 45.57 ± 3.42 | 26.35 ± 1.59 | 26.4 ± 1.56 | 30.1 |
| P1 | 7.5 | 4.99 ± 0.45 | 45.54 ± 2.39 | 26.11 ± 3.46 | 27.5 ± 3.25 | 30.5 |
| P2 | 9.0 | 6.09 ± 0.74 | 39.65 ± 3.84 | 26.28 ± 0.98 | 28.0 ± 3.25 | 41.7 |
| P3 | 10.0 | 6.40 ± 0.47 | 43.17 ± 2.85 | 26.28 ± 2.43 | 24.8 ± 1.28 | 44.6 |

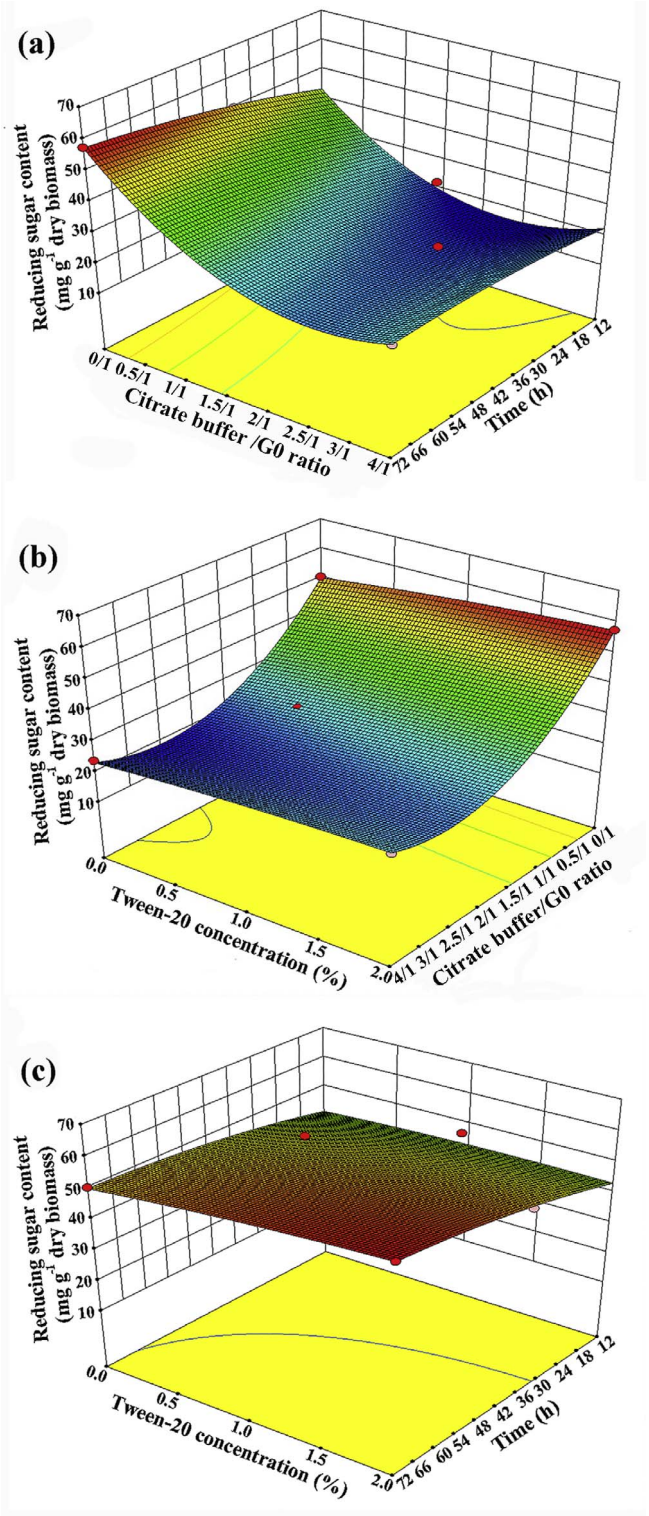


**Fig. 4.** Effects of pretreatment time, citrate buffer/G0 fermentation broth ratio and Tween-20 concentration on reducing sugar production using *Bacillus* sp. G0 via response surface methodology. (a) Effects of pretreatment time and citrate buffer/G0 fermentation broth ratio; (b) Effects of citrate buffer/G0 fermentation broth ratio and Tween-20 concentration; (c) Effects of pretreatment time and Tween-20 concentration.

not shown). Three parameters including pretreatment time, citrate buffer/G0 fermentation broth ratio and surfactant concentration (Tween-20) were used to optimize the production of reducing sugars via response surface methodology (Fig. 4). The reducing sugar production during the pretreatment process was finally fitted as the following equation:

**Table 3**
ANOVA of the quadratic model coefficient of reducing sugar production.

| Source | Sum of squares | DF | Mean square | F value | Prob > F |
|---|---|---|---|---|---|
| Model | 2854.25 | 9 | 317.14 | 24.16 | < 0.0001 |
| A | 128.27 | 1 | 128.27 | 9.77 | 0.0108 |
| B | 1538.00 | 1 | 1538.00 | 117.17 | < 0.0001 |
| C | 23.32 | 1 | 23.32 | 1.78 | 0.2122 |
| AB | 35.20 | 1 | 35.20 | 2.68 | 0.1325 |
| AC | 13.16 | 1 | 13.16 | 1.00 | 0.3403 |
| BC | 3.95 | 1 | 3.95 | 0.30 | 0.5954 |
| $A^2$ | 12.45 | 1 | 12.45 | 0.95 | 0.3532 |
| $B^2$ | 719.96 | 1 | 719.96 | 54.85 | < 0.0001 |
| $C^2$ | 2.34 | 1 | 2.34 | 0.18 | 0.6815 |
| Residual | 131.26 | 10 | 13.13 | – | – |

$R^2$ = 0.9560; Adj $R^2$ = 0.9165; A: Time; B: the ratio of citrate buffer and G0 fermentation broth; C: Tween-20 concentration; DF: Degree of freedom.

Reducing sugars (mg $g^{-1}$ dry biomass) = 50.27337 + 0.33347 [Time] − 26.45334 [Citrate buffer/G0] + 2.14390 [Tween-20] − 0.043353 [Time] × [Citrate buffer/G0] + 0.046154 [Time] × [Tween-20] − 0.44681 [Citrate buffer/G0] × [Tween-20] − 2.01204E-003 $[Time]^2$ + 4.86500 $[Citrate\ buffer/G0]^2$ – 0.80182 $[Tween\text{-}20]^2$.

The results of the analysis of variance (ANOVA) revealed that the model was significant since the model F-value was up to 24.16 (Table 3). The larger the number of the model F-value, the more significant is the corresponding coefficient (Qu et al., 2017). This large "model F-value" indicated that there was only a 0.01% chance to occur due to noise. The high value of the correlation coefficient ($R^2$ = 0.9560) illustrated a significant correlation between the experimental values and the predicated values and only 4.40% of the total variation cannot be explained by the model (Peng et al., 2014). In addition, the value of "Pro > F" less than 0.0500 indicated that model terms are significant. Estimated regression coefficient analysis showed that the ratio of citrate buffer and G0 fermentation broth had the most effects on the reducing sugar production than pretreatment time and Tween-20 concentration (Table 4). According to the optimal model, the maximum reducing sugars of 62.6 mg $g^{-1}$ dry biomass were obtained when the ratio of citrate buffer and G0 fermentation broth at 0.34, the pretreatment time at 100 h and Tween-20 concentration at 1.73% were used (Fig. 4).

### *3.4. Increasing digestibility of Miscanthus using the optimal pretreatment conditions*

FTIR spectra of G0 and G0 + Tween-20 pretreated biomass were recorded in the 4000–600 $cm^{-1}$ region. In *Miscanthus*, the bands at 3436 $cm^{-1}$ are related to stretch O—H groups and at 2850 $cm^{-1}$ C—H stretching of methyl, methylene or methane group. The signal at 1734 $cm^{-1}$ indicates C═O stretching of unconjugated ketone and carboxyl group and the absorbance at 1630 $cm^{-1}$ means the bending of

**Table 4**
Estimated regression coefficient for reducing sugar production.

| Factor | Estimate | DF | Standard error | 95% CI Low | 95% CI High | VIF |
|---|---|---|---|---|---|---|
| Intercept | 24.02 | 1 | 1.93 | 19.71 | 28.32 | – |
| A | 3.39 | 1 | 1.09 | 0.97 | 5.81 | 1.07 |
| B | −11.95 | 1 | 1.10 | −14.41 | −9.49 | 1.03 |
| C | 1.47 | 1 | 1.11 | −0.99 | 3.94 | 1.05 |
| AB | −2.28 | 1 | 1.39 | −5.37 | 0.82 | 1.05 |
| AC | 1.38 | 1 | 1.38 | −1.70 | 4.47 | 1.05 |
| BC | −0.78 | 1 | 1.43 | −3.96 | 2.39 | 1.05 |
| $A^2$ | −1.81 | 1 | 1.86 | −5.95 | 2.33 | 1.07 |
| $B^2$ | 14.90 | 1 | 2.01 | 10.42 | 19.38 | 1.07 |
| $C^2$ | −0.80 | 1 | 1.90 | −5.03 | 3.43 | 1.09 |

A: Time; B: the ratio of citrate buffer and G0 fermentation broth; C: Tween-20 concentration; DF: Degree of freedom; CI: Confidence internal; VIF: Variance inflation factor.

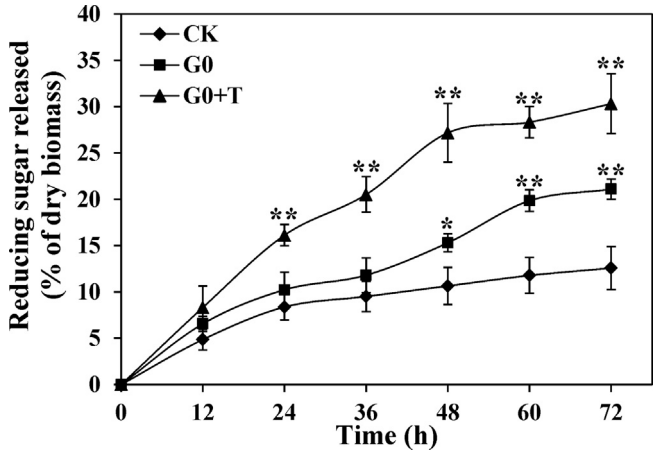


**Fig. 5.** Reducing sugars released from G0-pretreated *Miscanthus* using the optimal pretreatment parameters after enzymatic hydrolysis. Values represent mean ± SDs (n = 4). Different asterisks indicate significant differences at $*p < 0.05$ and $**p < 0.01$.

absorbed residual water. The peaks ranging from 1605 to 1253 $cm^{-1}$ are mainly associated with the change of lignin and aromatic ring, while the range of 1200–1172 $cm^{-1}$ and 1127–1094 $cm^{-1}$ correspond to C—O—C stretching at β-glucosidic linkages and C—O, C—C or C—OH bending in cellulose and hemicellulose, respectively. The C—O—C vibrations at β-glucosidic linkages in hemicellulose and cellulose happen in the peak of 898 $cm^{-1}$ (El Hage et al., 2009; Wang et al., 2010). After G0 pretreatment, the hemicellulose and cellulose related peaks around 3436, 2850, 1734, 1630, 1200, 1094 and 898 $cm^{-1}$ were lower than the untreated *Miscanthus*, especially in the presence of surfactant, while the absorption bands in the region of 1605–1253 $cm^{-1}$ were almost unaffected. The results showed that the physical and chemical structures of cellulose and hemicellulose were changed after pretreatment by G0. The pretreated *Miscanthus* by G0 + Tween-20 showed a rough, fragile and paralyzed surface compared to that of untreated *Miscanthus* by SEM analysis, thus further confirming the structural damage of cell wall compositions caused by bacterial pretreatment.

The total reducing sugars released from *Miscanthus* pretreated by G0 and G0 + Tween-20 were significantly increased about 1.5 and 3-fold compared to that of untreated *Miscanthus*, respectively, after 72 h of enzymatic saccharification (Fig. 5). The higher reducing sugars released from G0 + Tween-20 pretreatment *Miscanthus* can be explained as the follows: First, the higher xylanase produced by G0 caused efficient removal of hemicellulose biomass, which may improve the access of enzymes to cellulose, and ultimately led to the release of more sugar (Kumar et al., 2017). Second, surfactant Tween-20 helped to improve the enzymatic hydrolysis ability due to its adsorption on hydrophobic surfaces mainly composed of lignin fragments (Kaya et al., 1995). These was accord with that addition of Tween 20, a non-ionic surfactant significantly improved the hydrolysis of wheat straw by the enzymatic mixture (Tabka et al., 2006). Third, enzymes used in the hydrolysis process may be absorbed by the lignin, which reduced the amount of active enzyme available for hydrolysis (Bhagia et al., 2017; Kaar and Holtzapple, 1998). The protein produced by strain G0 may prevent this loss by blocking the surface of lignin and thus improving the utilization of available enzymes (Ferraz et al., 2017).

### 3.5. Mass balance

To track carbohydrate degradation of *Miscanthus* pretreated by the optimal pretreatment process, a mass balance was summarized including lignocellulosic enzyme production, pretreatment and enzymatic hydrolysis (Fig. 6). The maximum FPase, CMCase and xylanase activities in G0 strain were 2.2, 11.8 and 191.4 IU $g^{-1}$ dry biomass, respectively. After pretreatment, the solid recovery was up to 93.5% and the glucan content was 27.5% higher than that of untreated *Miscanthus*. The results almost were equivalent with the methods of alkali, acid and ammonia fiber expansion (AFEX) pretreatment (Liu et al., 2015; Xu et al., 2012; Zhong et al., 2009). The xylan content decreased by 45.2% and this is more sustainable to perform the fermentation process because most commonly used industrial fermentation microorganisms ferment glucose more efficiently than xylose (Wang et al., 2010). The total reducing sugars including 3.05 g from enzymatic hydrolysis and 0.61 g from the liquid per 10 g of pretreated *Miscanthus* were 2.9-fold higher than that of untreated *Miscanthus*.

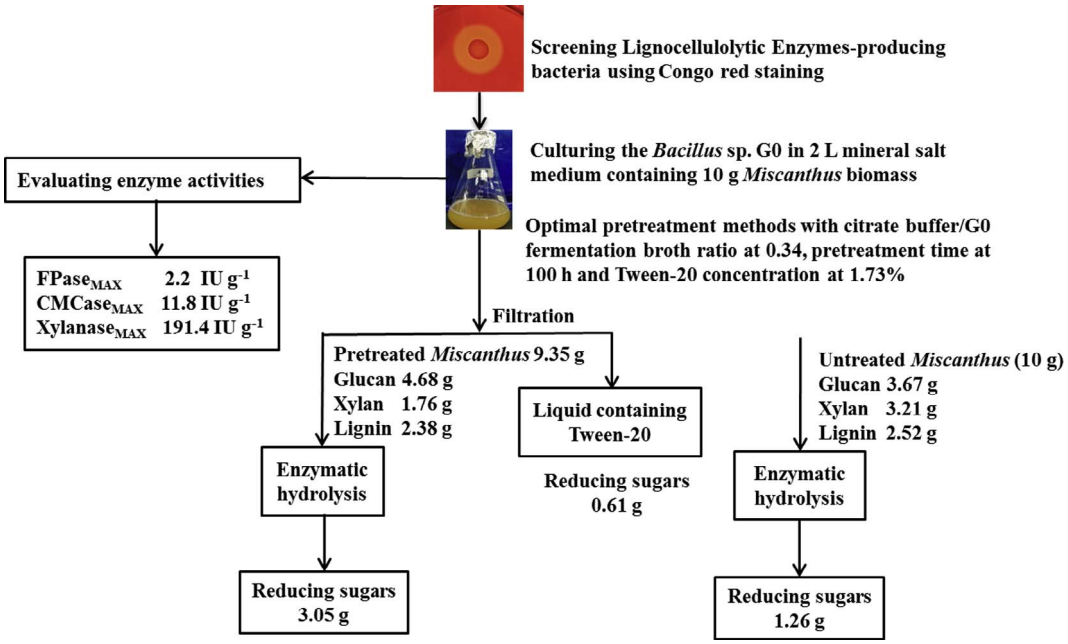


**Fig. 6.** Flow chart of experiments investigated the effects of *Bacillus* sp. G0 on lignocellulosic enzymes production, the removal of xylan and enzymatic hydrolysis of *Miscanthus* in the optimal pretreatment conditions.

## 4. Conclusions

An effective bacterial pretreatment method for increasing *Miscanthus* digestibility was investigated. After a two-step processing procedure (xylanase production using *Miscanthus* as carbon source followed by directly culturing the fermentation broth under the optimal temperature and pH), the cellulase accessibility was increased in pretreated *Miscanthus* accompanied by the reduction of hemicellulose content and CrI. The pretreatment parameters were further optimized via response surface methodology using *Bacillus* sp. G0 according to the two-step processing procedure. The highest reducing sugars released from *Miscanthus* pretreated under optimal conditions were up to 305 mg $g^{-1}$ dry biomass after 72 h of enzymatic saccharification.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2017.09.034.

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