Mutagenesis of *Aspergillus aculeatus* by ^{60}Co-γ Irradiation for High Production of Potential ILs-Tolerant Cellulase

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Ionic liquids (ILs) are effective solvents for lignocellulose pretreatment. Enzymatic saccharification converts pretreated lignocelluloses into valuable products, and IL-tolerant cellulase improves the enzymatic efficiency and the reuse of ILs. In this study, a fungal strain with a relatively high cellulase production was isolated and identified as *Aspergillus aculeatus* G1-3. The high production of β-glucosidase (1.943 U per mL), CMCase (1.303 U per mL), and FPase (0.165 U per mL) was obtained using corn stover as the carbon source and peptone as the nitrogen source. The results were obtained at pH 8.0 and 30 °C with an inoculation size of 3% (volume per volume) for 7 days. A mutant strain *Aspergillus aculeatus* P6 with β-glucosidase (7.023 U per mL), CMCase (1.543 U per mL), and FPase (0.098 U per mL) was obtained by ^{60}Co-γ irradiation. The cellulase activity was measured at pH 5.0 and 60 °C for enzymatic hydrolysis. The cellulase from mutant strains was stable in different concentrations of 1-ethyl-3-methylimidazolium acetate. Enzymatic saccharification of the original corn stover and ILs-pretreated corn stover was successfully performed with high sugar yields. The mutant strains of *Aspergillus aculeatus* have great potential for their further application in the conversion of lignocellulosic biomass into biofuels.

Keywords: Lignocellulosic biomass; Cellulase; Mutagenesis; Saccharification

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INTRODUCTION

Lignocellulosic biomass is one of the most abundant and renewable resources on earth. The bioconversion of biomass into biofuels has attracted increasing attention (Taha *et al*. 2015). Lignocellulosic biomass contains three main components: lignin, hemicelluloses, and cellulose. Those components form a complex and recalcitrant structure. Generally, enzymatic hydrolysis of cellulose is a crucial process in converting lignocelluloses into monomeric sugars or other high value-added products for biofuels.

A variety of microorganisms such as *Trichoderma reesei*, *Aspergillus niger*, *Penicillium oxalicum*, *Clostridium*, and *Bacteroides* can produce cellulolytic enzymes (Wenzel *et al.* 2010; Sun *et al.* 2018). Cellulase is a collection of multiple enzymes, of which there are three main components. Among these are endo-β-1-4-glucanase that cleaves the internal β-1,4 linkages of cellulose chains to create new reducing and non-reducing ends, exo-β-1-4-glucanase or cellobiohydrolase that removes disaccharide cellobiose from the non-reducing end of cellulosic chain, and β-glucosidase that hydrolyzes

the newly produced cellobiose and short chain cellodextrin units into monomeric glucose units (Gusakov 2011; Singhania *et al.* 2013; Paramjeet *et al.* 2018). *T. reesei* is a well-known source of cellulolytic enzymes for lignocellulose hydrolysis in industrial applications (Shuster and Schmoll 2010). However, the low β-glucosidase activity of *T. reesei* limits the hydrolysis efficiency (Baba *et al*. 2015). The addition of *Aspergillus* sp., such as *A. niger* and *A. terreus* with a high β-glucosidase activity, has gained much attention (Sohail *et al.* 2009).

Although enzymatic saccharification is a promising technology, the recalcitrant structure of lignocelluloses and cellulose crystallinity limit the enzymatic efficiency. Various approaches have been studied for the pretreatment of lignocellulosic biomass such as steam explosion, ammonia fiber expansion, and organic solvent-pretreatment (Treebupachatsakul *et al.* 2016). Roger *et al.* (2002) found a series of ionic liquids (ILs) containing various cations and anions, which could form hydrogen bonds with cellulose to improve cellulose solubility. ILs-pretreatment could remove partial lignin fractions from lignocelluloses and reduce the cellulose crystallinity to increase the enzymatic surface area (Zhu *et al.* 2018).

Commercially available enzymes are not compatible with the residue of ILs after ILs-pretreatment, leading to an inefficient subsequent saccharification (Peng *et al*. 2015). A large amount of water is required to remove any residue of ILs. Therefore, ILs-tolerant cellulase is necessary to improve the enzymatic efficiency. This is to reduce the extra water washing and to improve the reuse of ILs for industrial application.

In this study, a fungal strain with a high cellulase activity was isolated and identified. The optimal conditions for cellulase production including carbon and nitrogen sources, pH and inoculation size, temperature, and incubation time were studied. Furthermore, a series of mutant strains were obtained from the ^{60}Co-γ irradiation. The effects of pH and temperature on the cellulase activities during the enzymatic hydrolysis were determined. The ILs tolerance of cellulase from the wild and mutant strains were investigated. The enzymatic hydrolysis of corn stover and ILs-pretreated corn stover by the wild and mutant strains was evaluated.

MATERIALS AND METHODS

Sample collection

The surface soil (1 to 5 cm) was collected from a local corn farm in Jining (Longitude 115° 54', Latitude 34° 25'), Shandong province, China, and stored at 4 °C. Corn stover and rice straw were provided by the Xiangfang farm in Harbin, China; they were dried, ground, and passed through 40 mesh sieves. Wheat bran was provided by the Xiangfang Flour Factories of Harbin in China. Chemicals were purchased from Aladdin Reagent Co. LTD (Shanghai, China).

Isolation of Cellulase Producing Strains

A total of 10 g of the soil samples was resuspended and serially diluted in sterile distilled water, and 100 μL of the diluted sample was spread on PDA agar plates, which were incubated at 30 °C for 5 days. The colonies of various fungi were isolated. A single colony was incubated on CMC medium (K_2HPO_4, 1.0 g; NaCl, 0.5 g; $(NH_4)_2SO_4$, 2.0 g; $MgSO_4$, 0.5 g; CMC-Na, 10 g; and agar, 15 g) at 30 °C for 3 days. All plates were stained

with Congo-red solution (0.1%, weight per volume) for 15 min and destained with 1 M NaCl to isolate the cellulase producing strains.

The fungal strain was further incubated in PDA medium at 30 °C for 3 days with shaking at 180 rpm. Next, 1 mL of the suspension (4 to 6 mg of dry mycelia per mL) was added into 100 mL of Hutchinson inorganic salt medium (KH_2PO_4, 1.0 g; $MgSO_4$, 0.3 g; NaCl, 0.1 g; $CaCl_2$, 0.1 g; and $FeCl_3$, 0.01 g) with cover stover of 1% (weight per volume) and peptone of 0.2% (weight per volume). It was incubated at 30 °C for 5 days with shaking at 180 rpm. The resulting solution was centrifuged at 10,000 rpm for 10 min at 4 °C to give the crude enzyme.

Determination of Enzyme Activity

The enzymatic activity based on assays for carboxymethyl cellulase (CMCase), β-glucosidase, and filter paper cellulase (FPase) was determined by measuring the release of reducing sugars. This was accomplished with 0.5 mL of the crude enzyme from the strains in 1.5 mL of the sodium citrate buffer. The buffer had pH 4.8 at 50 °C, and the measurement had an addition of 1.5 mL of carboxymethyl cellulose (1%, weight per volume) for 30 min, 1.5 mL of D(-)-Salicin (0.5%, weight per volume) for 30 min, or Whatman No. 1 filter paper (1.0 by 6.0 cm) for 60 min, respectively. The reducing sugar was measured according to the dinitrosalicylic acid (DNS) method (Miller 1959; Papa *et al.* 2017). Standard curves were prepared under assay conditions for glucose. One unit of cellulase activity was defined as the amount of enzyme that releases 1 μmol of reducing sugar per minute.

Molecular Identification

DNA extraction of the strain was conducted using a DNA kit according to the manufacturer's instructions (OMEGA, Norcross, GA, USA). The rDNA was amplified by a polymerase chain reaction (PCR) using the universal primer pair ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCGCCTTATTGATATGC-3'). The PCR products were sequenced in the Huada gene company (Beijing, China). The sequencing results were compared using the Basic Local Alignment Search Tool (BLAST) program on NCBI. A phylogenetic tree was constructed using the neighbor-joining model of the MEGA 5.0 program.

Optimization of the Conditions for Enzyme Production

To identify the optimal conditions for the cellulase production, carbon source and nitrogen source, initial pH and inoculation size, and temperature and incubation time were evaluated. The strain was incubated in 100 mL of the PDA medium on a rotary shaker at 180 rpm. The carbon and nitrogen sources were examined with 1% (volume per volume) of inoculation at 30 °C and at pH 7.0 for 5 days. The initial pH and inoculation size were investigated at 30 °C for 5 days with corn stover and peptone. The temperature and incubation time were determined with 3% (volume per volume) of inoculation at pH 8.0 with corn stover and peptone. Following that, the solution was centrifuged at 10,000 rpm for 10 min. The resulting supernatant was further utilized for the enzyme assay. Each experiment was conducted in triplicate.

Mutagenesis of the Strain

The spore suspension was prepared by gradient dilution method and (10^5 to 10^6 per mL) was exposed to different doses of the ^{60}Co-γ irradiation with 1000, 1500, 2000, 2500, or 3000 Gray for 5 min by a Mark V irradiator (1.5×10^5 Ci) at Heilongjiang Academy of

Agricultural Sciences. The spore suspension was diluted into 100-fold, and then 0.1 mL of diluted solution was spread on the PDA medium. It was incubated at 30 °C for 3 days to isolate the mutant strains.

Optimization of pH and Temperature for Enzymatic Hydrolysis

Each reaction was carried out in 50 mmol of the citrate buffer at 50 °C for 1 h at a pH value ranging from 4 to 6 that was adjusted by the citrate buffer. Each reaction was performed in 50 mmol citrate buffer by varying temperatures in the range of 30 °C to 75 °C at pH 5.0 for 1 h. Each experiment was conducted in triplicate.

Determination of the Ionic Liquid Tolerance of Cellulase

1-Ethyl-3-methylimidazolium acetate [EMIM]Ac was synthesized using a published method (Sun *et al*. 2018). The crude enzyme was mixed with the citrate buffer at pH 5.0 with the addition of [EMIM]Ac in the range of 0.0%, 2.5%, 5.0%, 7.5%, 10.0%, and 15.0% (weight per volume). The solution was cultured for 1 h at 50 °C. Cellulase activity in [EMIM]Ac was determined. Each experiment was conducted in triplicate.

Enzymatic Saccharification of Corn Stover

A total of 1 g of corn stover was pretreated with 20 g of [EMIM]CH_3COOH at 120 °C for 3 h. After that, 50 mg of the original corn stover or [EMIM]CH_3COOH-pretreated corn stover was mixed with the crude enzyme solution or commercial cellulase at 1 U of FPase activity. The reaction mixture was then replenished to 20 mL using the citrate buffer at pH 5.0. It was stirred at 60 °C and 180 rpm for 72 h. The commercial cellulase (*T. reesei* ATCC26921) was used as a positive control. The reducing sugars were measured by the 3, 5-dinitrosalicylic acid (DNS) method. Each experiment was conducted in triplicate.

RESULTS AND DISCUSSION

Isolation and Identification of Cellulase Producing Strains

The Congo-red staining method was employed for the preliminary screening of cellulase producing strains (Wood *et al*. 1988). The fungal strains with discoloration zones of Congo-red were isolated as the positive microbes, indicating their ability to secrete cellulase. One of the isolates with high enzyme activities was selected for further studies.

ITS sequences that are highly conserved sequences are useful for phylogenetic analysis for the identification of microorganisms. The isolate was identified as *A. aculeatus* G1-3 based on its ITS sequence of DNA, as shown in the phylogenetic tree (Fig. 1). The obtained nucleotide sequence was submitted to the NCBI GenBank with an under-accession No. of MN163019. A variety of *Aspergillus* sp including *A. aculeatus*, *A. niger*, *A. oryzae, A. nidulans, A. fumigatus*, and *A. terreus* have been reported to produce cellulose (Sandhu *et al.* 2012; Kaur *et al.* 2013; Oberoi *et al.* 2014). *A. aculeatus* possesses the capability to produce various cellulase, especially high β-glucosidase activity (Treebupachatsakul *et al*. 2016).

Optimization of the Conditions for Enzyme Production

Effects of carbon sources and nitrogen sources

The fungal cellulase production is mainly dependent on the nature of carbon sources and nitrogen sources (Panagiotou *et al*. 2003). Generally, cellulose-rich substrates

as carbon sources could induce cellulase production in fermentation (Kim *et al*. 2014). As shown in Fig. 2a, corn stover and wheat bran were effective carbon sources for *A. aculeatus* G1-3. Particularly, the maximum cellulase activity including CMCase, β-glucosidase, and FPase was observed when corn stover was used as the sole carbon source. However, the negligible cellulase activity was observed with the addition of CMC and α-Cellulose (Fig. 2a). This is probably because of lignocellulosic materials being more accessible for microbes compared with commercial polymeric materials (Wood *et al.* 1988; Liu *et al.* 2011). The inexpensive and readily available corn stover was preferred for industrial utilization.

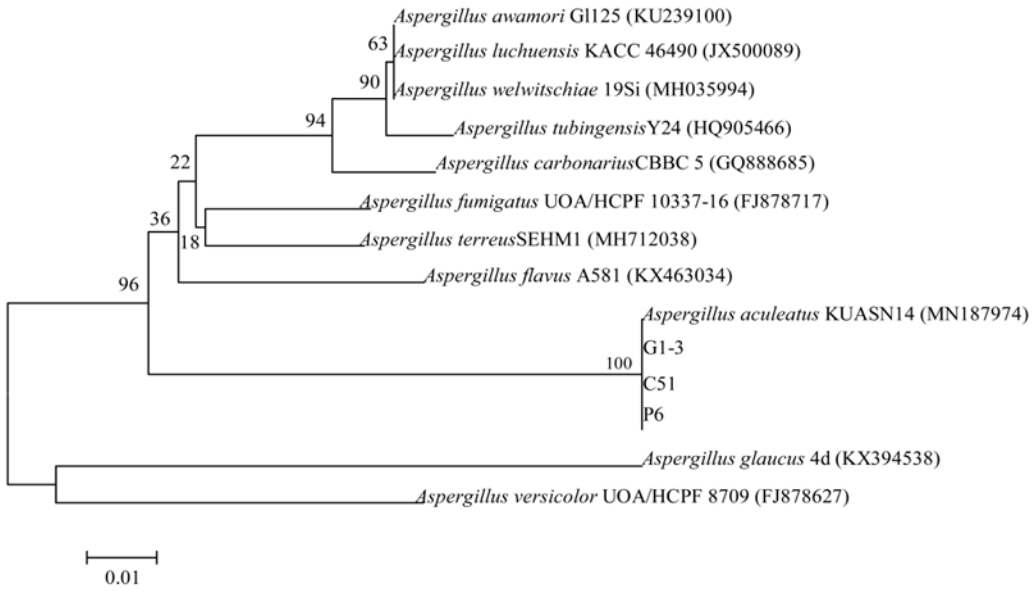

Fig. 1. Phylogenetic relationship of fungal strains

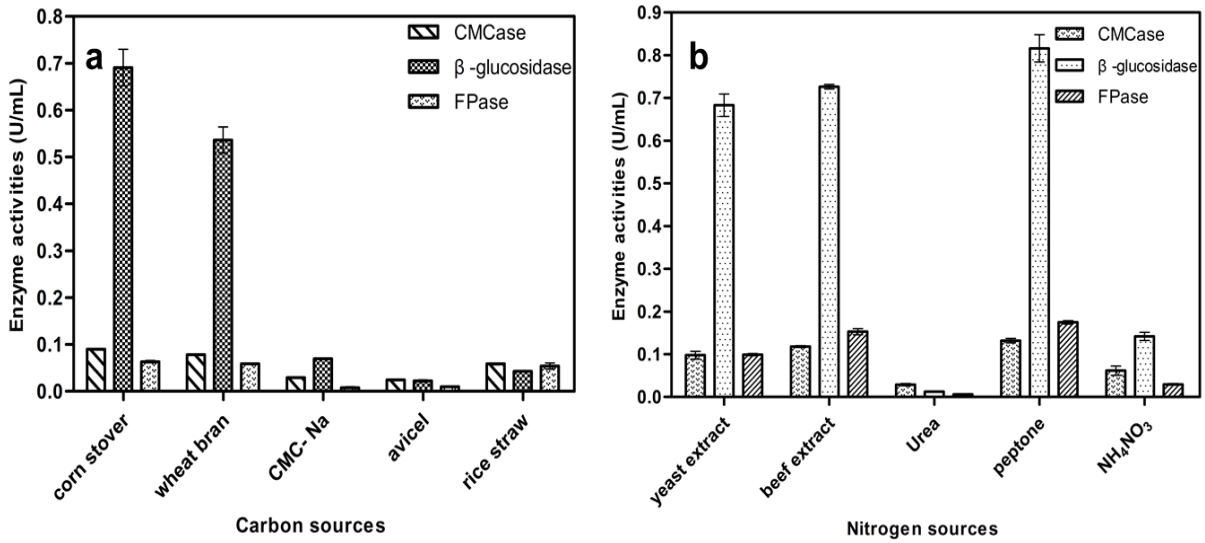

Fig. 2. Effects of carbon sources (a) and nitrogen sources (b)

The nitrogen source that is supplemented in organic or inorganic form depends on different microorganisms. As illustrated in Fig. 2b, a high cellulase activity including CMCase, β-glucosidase, and FPase was observed using peptone as the sole nitrogen source. In contrast, a low cellulase activity was observed when NH_4NO_3 or urea was used. Previous studies showed that organic nitrogen sources resulted in a higher production of cellulase than inorganic nitrogen (Yeoh *et al.* 1986; Akiba *et al.* 1995). In this study, organic nitrogen sources including yeast extract and beef extract were more effective than inorganic nitrogen sources such as NH_4NO_3 and urea. The effective peptone was selected to be the optimal nitrogen source in the following study.

Effects of inoculation size and temperature

An appropriate inoculation size is important for substrate utilization in order to increase fungal growth and enzyme production. As described in Fig. 3a, as the inoculation size increased, the strain grew rapidly with a high cellulase production. The maximum activities of β-glucosidase and FPase were obtained with the inoculation concentration of 3% (volume per volume). When the inoculation size was increased to 5% (volume per volume), the CMCase activity improved. A balance between nutrients and growing cells is necessary for the optimum enzyme production (Ramachandran *et al*. 2004).

A low temperature may induce low metabolic activities of microorganisms. However, a high temperature may cause thermal denaturation of the metabolic pathway of enzymes, resulting in a low cellulase production. As presented in Fig. 3b, a high production of CMCase of *A. aculeatus* G1-3 was achieved at 20 °C. The high production of β-glucosidase and FPase was obtained at 30 °C and 35 °C. When the temperature was increased more than 40 °C, enzyme production was reduced. The optimum temperature for cellulase production is 25 to 28 °C for *Trichoderma* sp. (Chandra *et al*. 2010) and 30 °C for *A. niger* (Hanif *et al*. 2004).

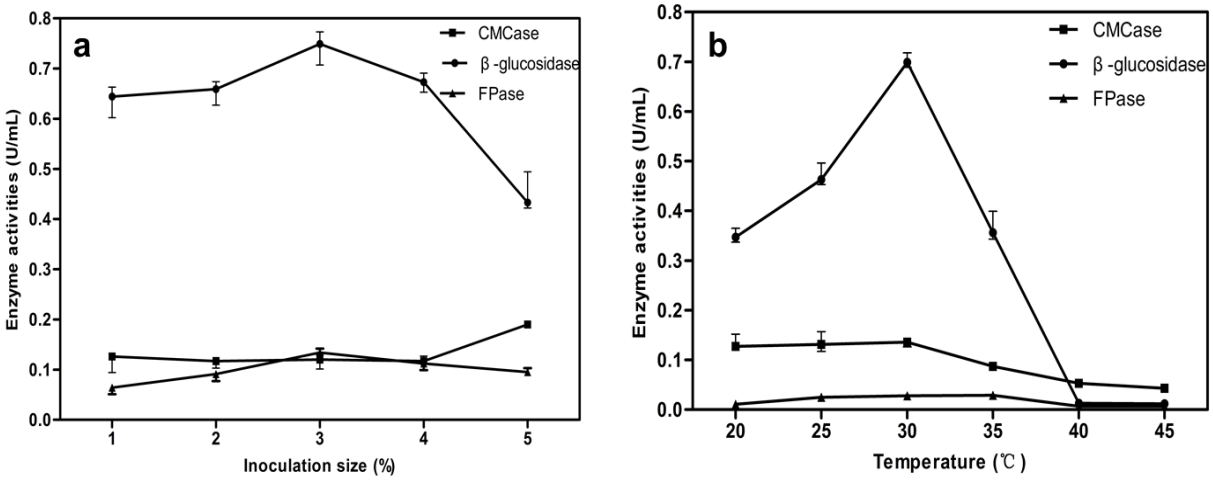

Fig. 3. Effects of inoculation size (a) and temperature (b)

Effects of initial pH and incubation time

Enzyme systems of microbes could be affected by the initial pH of the medium. As presented in Fig. 4a, the cellulase production of *A. aculeatus* G1-3 was increased with the increase of the pH value from 3.0 to 11.0. The maximum activity of CMCase, β-glucosidase, and FPase was observed at pH 8.0. Commonly, the preference for acidic pH has been found for cellulase producing microbes. A pH range of 4.5 to 4.8 is favorable for

cellulase production by *T. reesei* and *A. phoenicis* (Yeoh *et al*. 1986). The optimal pH for cellulase production from *A. niger* was found to be between 6.0 and 7.0 (Akiba *et al*. 1995). *Aspergillus* sp. were reported to grow and metabolize well in the acidic pH medium between 3.0 and 5.0 (Li *et al*. 2013). In this work, broad pH values (7.0 to 11.0) were generated by *A. aculeatus* G1-3, which was obviously different from other *Aspergillus* sp. This indicated their potential application in degradation of corn stover, particularly for alkali-treated samples.

A considerable variation in cellulase production was observed during different incubation times (Fig. 4b). Initially, the strain grew vigorously and the cellulase yield was improved when the incubation time was increased from 4 to 7 days. After 7 days, β-glucosidase production increased remarkably, whereas the CMCase production decreased. The high activities of β-glucosidase, CMCase, and FPase were obtained after 12 days, 7 days, and 8 days of incubation, respectively. As reported, the high CMCase activity was observed after 5 days for *A. japonicas* (Vega *et al*. 2014). The highest FPase and CMCase activities were found after day 8 and 10 for *Myceliophthorasp* (Badhan *et al*. 2007). No remarkable difference was observed for the incubation time between *A. aculeatus* G1-3 with other reported fungi.

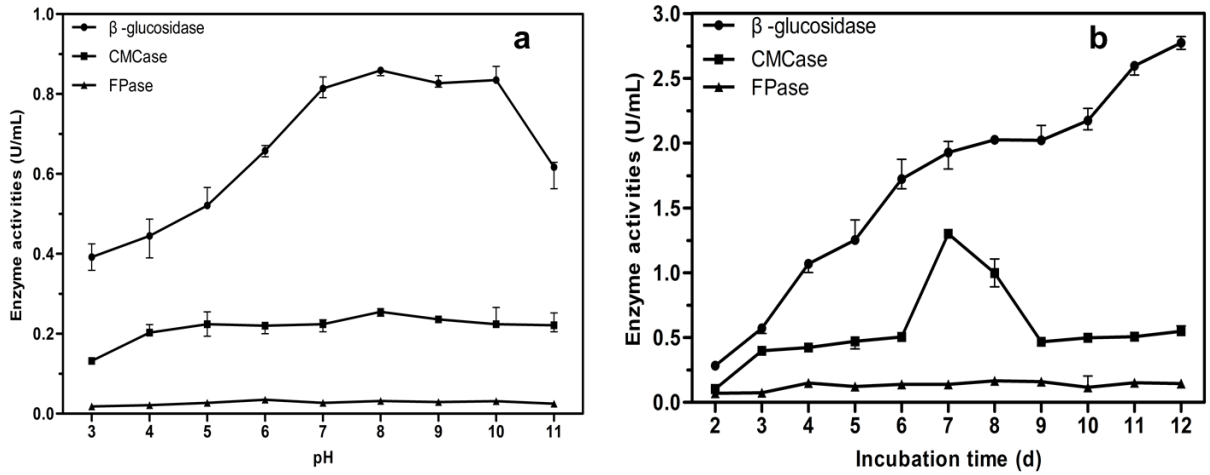

Fig. 4. Effects of pH (a) and incubation time (b)

Therefore, a relatively high production of FPase, CMCase, and β-glucosidase for the wild *A. aculeatus* G1-3 strain were obtained at 0.165 U per mL, 1.303 U per mL, and 1.943 U per mL, respectively. This was obtained under the optimal conditions using corn stover and peptone at pH 8.0 and a temperature of 30 °C with an inoculation size of 3% (volume per volume) for 7 days.

Mutagenesis of the Wild Strain

^{60}Co-γ irradiation is an effective approach that has been widely used for plants mutagenesis (Miao *et al*. 2015). Currently, it was successfully utilized in microorganism mutagenesis in order to improve their metabolism products (Engel *et al*. 2010). Initially, the spore suspension treated by ^{60}Co-γ ray at the dose of 2500 Gray was coated on a PDA plate. A total of 1531 mutants were isolated and purified, and cellulase activity was determined by shake flask fermentation.

Table 1. ^{60}Co γ Mutagenesis for *A. aculeatus* G1-3

Strain	β-Glucosidase Activity (U/mL)	Relative Activity (%)	CMCase Activity (U/mL)	Relative Activity (%)	FPase Activity (U/mL)	Relative Activity (%)
G1-3	1.943 ± 0.3	100	1.303 ± 0.3	100	0.165 ± 0.3	100
P6	7.023 ± 0.1	361 ± 0.8	1.543 ± 0.1	118 ± 0.8	0.094 ± 0.1	59 ± 0.8
C22	2.618 ± 0.1	134 ± 0.8	0.782 ± 0.1	60 ± 0.8	0.237 ± 0.1	143 ± 0.8
C8	2.985 ± 0.2	153 ± 1.6	0.481 ± 0.2	37 ± 1.6	0.061 ± 0.2	37 ± 1.6
C58	2.145 ± 0.2	110 ± 1.6	0.741 ± 0.2	57 ± 1.6	0.183 ± 0.2	111 ± 1.6
C29	3.131 ± 0.4	161 ± 3.2	0.179 ± 0.4	14 ± 3.2	0.109 ± 0.4	66 ± 3.2
C43	2.299 ± 0.4	118 ± 3.2	0.106 ± 0.4	8 ± 3.2	0.254 ± 0.4	153 ± 3.2
C42	2.815 ± 0.2	144 ± 1.6	0.514 ± 0.2	39 ± 1.6	0.129 ± 0.2	78 ± 1.6
C96	1.738 ± 0.2	89 ± 1.6	0.643 ± 0.2	49 ± 1.6	0.178 ± 0.2	107 ± 1.6
C12A-1	3.115 ± 0.3	160 ± 2.4	0.340 ± 0.3	26 ± 2.4	0.104 ± 0.3	63 ± 2.4
C80	2.372 ± 0.3	122 ± 2.4	0.724 ± 0.3	56 ± 2.4	0.221 ± 0.3	134 ± 2.4
C18	3.093 ± 0.1	159 ± 0.8	0.501 ± 0.1	38 ± 0.8	0.059 ± 0.1	36 ± 0.8
C51	3.443 ± 0.2	177 ± 1.6	0.618 ± 0.2	47 ± 1.6	0.134 ± 0.2	81 ± 1.6

The cellulase production of a variety of dominant strains is shown in Table 1. A high β-glucosidase production was achieved with the mutant strains C51 (3.443 U per mL) and P6 (7.023 U per mL), which were 177%. This was 361% higher than that of the wild *A. aculeatus* G1-3 strain (1.943 U per mL). In addition, the mutant P6 and C58 strains displayed high CMCase production at 1.543 U per mL and 0.782 U per mL, remaining 118% and 57% compared with the wild strain (1.303 U per mL). The mutant C43 and C22 strains exhibited maximum FPase production at 0.254 U per mL and 0.237 U per mL, which were 153% and 143% higher than the wild strain (0.165 U per mL), respectively. The cellulase production of *A. aculeatus* P6 and *A. aculeatus* C51 were high and stable throughout 10 generations. The genomic DNA by mutagenesis possibly resulted in a remarkable alteration of the enzyme expression.

Cellulase Stability at Different pH and Temperature

Factors including pH and temperature on the cellulase activities of the wild and mutant strains during enzymatic hydrolysis were studied. As shown in Fig. 5, cellulase activities were improved when the pH was increased from 4.0 to 5.0.

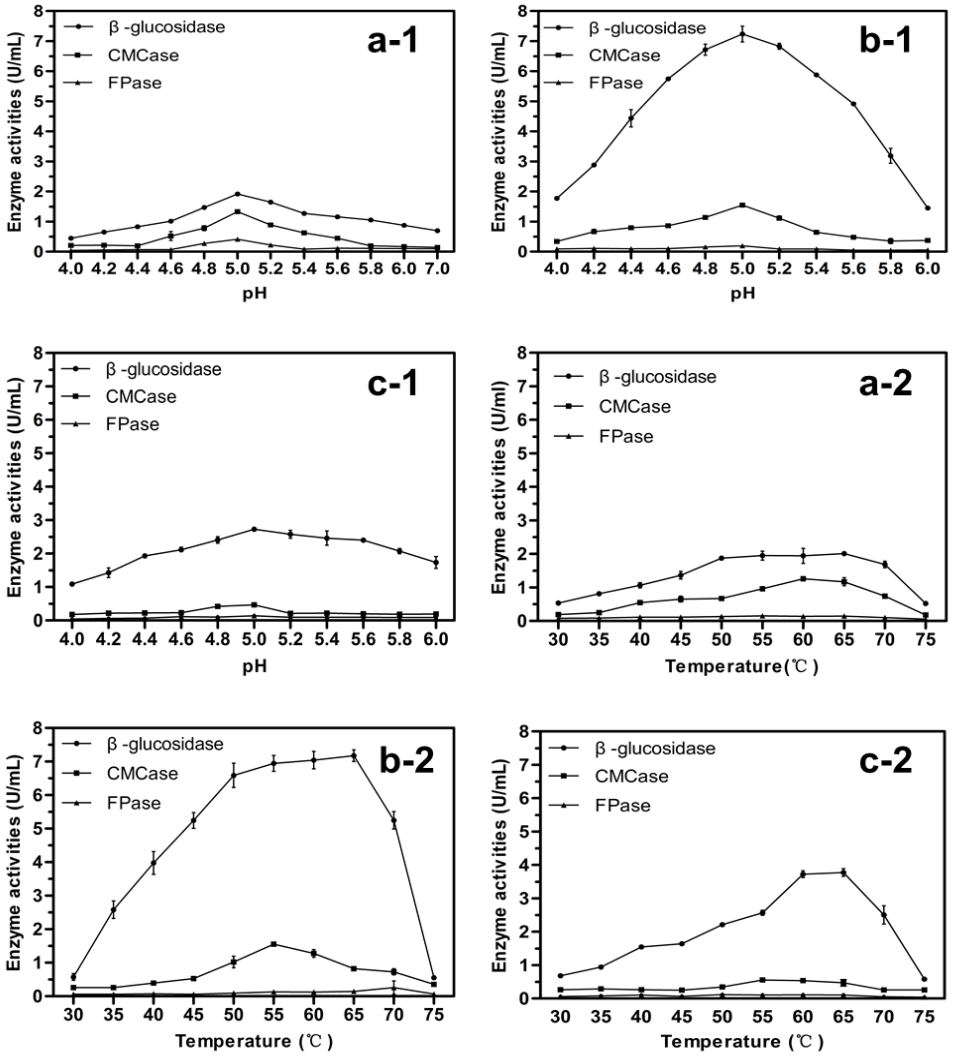

Fig. 5. Enzyme activities at different pH and temperatures levels for (a) *A. aculeatus* G1-3, (b) *A. aculeatus* P6, and (c) *A. aculeatus* C51

However, most cellulase activities were reduced after pH 5.0. This indicated cellulase from *A. aculeatus* was sensitive to pH during the hydrolysis process. Specifically, β-glucosidase retained a high activity in a pH range of 4.8 to 5.6 for the wild G1-3 strain (Fig. 5a-1) and in a pH range of 5.0 to 5.4 for the mutant P6 strain (Fig. 5b-1). Meanwhile, CMCase and FPase from both the wild and mutant strains were optimally active at pH 5.0. As reported, *A. fumigatus* showed an optimum FPase activity at pH 5.0 (Ang *et al*. 2013). Cellulase from *Melanocarpus sp.* was active between pH 4 to 6 with an optimal β-glucosidase activity at pH 5.0 (Kaur *et al*. 2007). Acidic cellulase are always desirable for the bioconversion of biomass, particularly when the β-glucosidase must work in consonance with *T. reesei* enzymes. *T. reesei* enzymes optimal activity was reported at pH 5.0 as well (Singhania *et al*. 2010). In this study, the optimal pH was observed at 5.0 for the wild and mutant strains.

The temperature stability of cellulase obtained from the wild and mutant strains is shown in Fig. 5. Initially, the cellulase activities were increased with the enhancement of the reaction temperature. All cellulase specimens were relatively stable in a temperature range of 55 to 70 °C. An optimal temperature of 65 °C was observed with high β-glucosidase activities for both the wild G1-3 strain (2.006 U per mL) and mutant P6 strain (7.177 U per mL). High CMCase activities were obtained at 60 °C for the G1-3 strain (1.261 U per mL) and at 55 °C for the mutant P6 strain (1.554 U per mL). FPase activities were stable in a temperature range of 55 to 65 °C. Currently, most of the commercial cellulase was reported to have optimal activity at 50 °C (Sukumaran *et al*. 2009). An optimal temperature of 60 °C was reported for cellulase hydrolysis by *A. fumigatus* (Ang *et al*. 2013). Similarly, a high cellulase activity was obtained from *A. heteromorphus* at 60 °C (Singh *et al*. 2009). Notably, β-glucosidase maintained good activity, although the temperature was increased to 70 °C in this study.

ILs-tolerance of Cellulase from the Wide Type and the Mutant Strains

ILs-tolerance of cellulase from the wild and mutant strains was investigated as illustrated in Fig. 6.

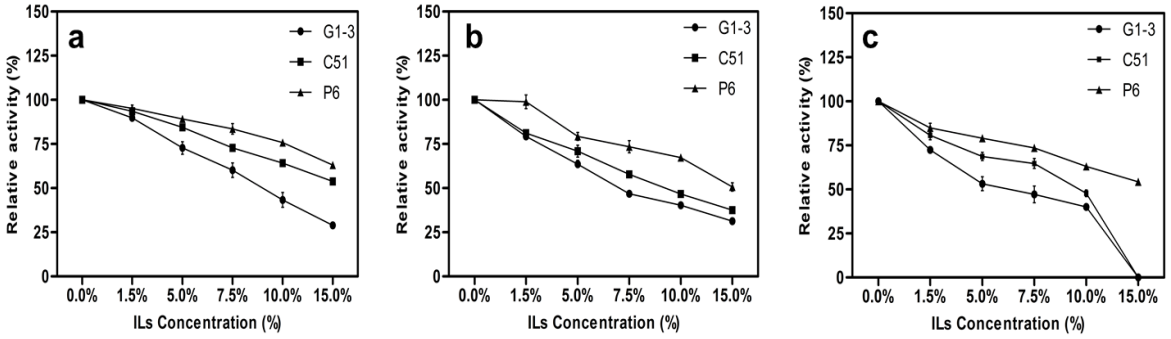

Fig. 6. ILs tolerance of cellulase (a) β-glucosidase, (b) CMCase, (c) FPase

Cellulase activity of each strain at 0% (weight per volume) of 1-ethyl-3-methylimidazolium acetate ([EMIM]CH_3COOH) is considered to be 100%. Obviously, ILs-tolerance of the cellulase from the mutant P6 and C51 strains were remarkably higher when compared to the wild G1-3 strain. As the concentration of [EMIM]CH_3COOH was 2.5% (weight per volume), the mutant P6 strain achieved a high enzymatic activity with β-glucosidase (95.1%), CMCase (98.9%), and FPase (85.9%). When the concentration of

[EMIM]CH_3COOH was increased to 15% (weight per volume), the mutant P6 strain maintained the enzymatic activity with β-glucosidase (63.1%), CMCase (50.6%), and FPase (53.1%). A previous study reported that β-glucosidase from *T. reesei* was almost undetectable at 1-ethyl-3-methylimidazolium acetate ([EMIM]CH_3COOH) concentration higher than 15% (volume per volume) (Xu *et al*. 2015). CMCase of *Fusarium oxysporum* BN isolated from the chemical contaminated areas could maintain 13% content in 10% (weight per volume) 1-allyl-3-methylimidazolium chloride [Amim][Cl] (Auxenfans *et al*. 2017). Therefore, ^{60}Co-γ irradiation was an effective approach for mutagenesis of the cellulolytic fungi for obtaining ILs-tolerant cellulase. Cellulase from the mutant P6 strain was much more stable with the ionic liquid ([EMIM]CH_3COOH) than the wild strain.

Enzymatic Saccharification of Corn Stover

The fungal cellulase activities of the wild type and mutant strains on the saccharification of the original corn stover and ILs-treated corn stover were studied by measuring the total reducing sugars (Hu *et al.* 2013; Zhang *et al.* 2015). After 72 h of the saccharification of the original corn stover, 9.42 mg per mL, 8.21 mg per mL, and 4.12 mg per mL of the reducing sugar were obtained from the crude enzyme of the P6 mutant strain, the C51 mutant strain, and the wild G1-3 strain, respectively (Fig. 7). In addition, the yields of the reducing sugar from [EMIM]CH_3COOH-pretreated corn stover were obviously higher than that of the untreated corn stover. The yields of the reducing sugar were obtained at 21.2 mg per mL and 20.07 mg per mL from the crude enzyme of the mutant P6 and C51 strain, respectively. The yield of the reducing sugar was obtained at 19.43 mg per mL from the crude enzyme of the wild G1-3 strain. The decrease of cellulose crystallinity and the effective removal of partial lignin and hemicellulose improved the cellulase accessibility during enzymatic hydrolysis to obtain more sugars (Chuanliang *et al.* 2011; Timilsena *et al.* 2013).

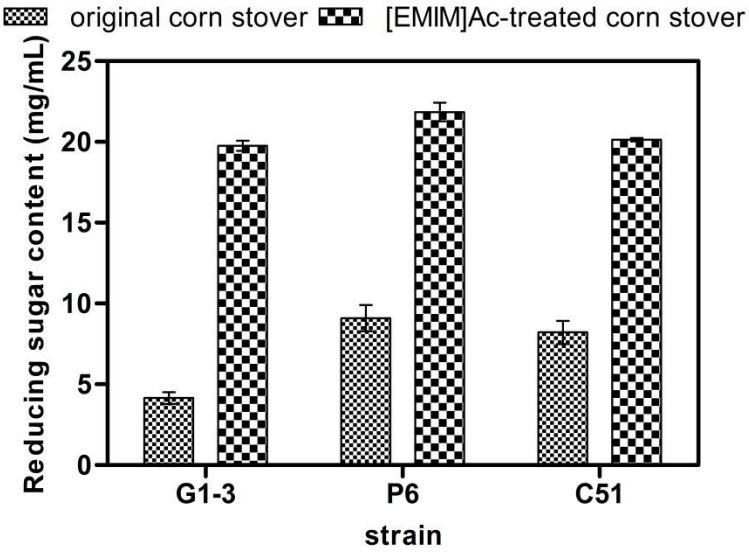

Fig. 7. Enzymatic saccharification

CONCLUSIONS

1. In this study, a fungal strain was newly isolated and identified as *Aspergillus aculeatus* G1-3. A variety of dominant strains were obtained from the mutagenesis by ^{60}Co-γ irradiation.
2. The mutant P6 strain displayed β-glucosidase (7.02 U per mL), CMCase (1.54 U per mL), and FPase (0.09 U per mL) activity. The mutant C51 strain exhibited β-glucosidase (3.44 U per mL), CMCase (0.62 U per mL), and FPase (0.13 U per mL). The optimal pH was observed at 5.0 for cellulase from both the wild G1-3 strain and the mutant P6 and C51 strains. The optimal temperature was observed at 65 °C for β-glucosidase and FPase, as well as 55 °C for CMCase.
3. The cellulase from the wild and mutant strains were relatively stable in the presence of [EMIM]CH_3COOH when compared to the current study. The saccharification of [EMIM]CH_3COOH-treated corn stover produced 19.4 mg per mL of reducing sugar from the crude enzyme of the wild G1-3 strain. It also produced 21.2 mg per mL and 20.1 mg per mL from the crude enzyme of the mutant P6 and C51 strains, respectively. This indicated its great potential for application in the conversion of lignocelluloses to biofuels.

ACKNOWLEDGMENTS

This work was financially supported by the Harbin science and Technology Innovative Talents Project (Grant No. 2017RAQXJ148) and Academic Backbone "Project of Northeast Agricultural University" (18XG26).

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Article submitted: March 12, 2020; Peer review completed: July 12, 2020; Revised version received and accepted: July 15, 2020; Published: July 23, 2020.
DOI: 10.15376/biores.15.3.6974-6988