

Screening of Highly Efficient Fungi for the Degradation of Lignocelluloses by Ionic Liquids-assisted Cellulase

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A successful conversion of lignocellulosic biomass into biofuel could be achieved through an ionic liquids (ILs)-pretreatment and an enzyme saccharification. In this study, the fungal strains with high cellulase productions were isolated and identified as *Penicillium janthinellum* FHH1 and *P. oxalicum* FLY4. A high cellulase production was obtained at pH 4.0 and 30 °C for *P. janthinellum* FHH1 with corn stalk and beef extract for 9 days and for *P. janthinellum* FHH1 with corn stalk and peptone for 7 days. A pH range between 4.0 to 6.0 and a temperature range from 55 °C to 60 °C were applicable for cellulase with high activities during hydrolysis. A high NaCl-stability and a relatively high ILs-stability were found for cellulase obtained from *P. oxalicum* FLY4. High yields of reducing sugar were obtained by enzymatic hydrolysis of the original corn stalk and ILs-pretreated corn stalk. *P. oxalicum* FLY4 has a great potential for ILs-assisted fungi in the degradation of lignocellulosic biomass into value-added products.

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INTRODUCTION

Lignocellulosic biomass is one of the most abundant and renewable resources on earth. The focus on the bioconversion of lignocellulosic biomass into biofuels has increased (Taha *et al.* 2015; Jatoi *et al.* 2021). Cellulose and hemicellulose, which comprise two-thirds of lignocelluloses, are known as the main feedstock for biofuel production. The recalcitrant nature of lignocelluloses and the high crystallinity of cellulose limit the effective utilization of cellulosic biomass. An effective approach to convert cellulose into a valuable product is enzymatic saccharification (Pandey *et al.* 2000; Kirk *et al.* 2002). However, the low efficiency and the high cost of cellulase are still the main challenges faced (Baba *et al.* 2015).

Cellulase plays an important role in the release of monomeric sugars from cellulosic biomass. A complete cellulase system includes endoglucanase (EG), cellobiohydrolase (CBH), and β -glucosidase (BGL). These enzymes hydrolyze the β -1,4-D-glucan bonds in the cellulose to release glucose, cellobiose, and oligosaccharides (Paramjeet *et al.* 2018). Numerous microbes, such as *Trichoderma* sp., *Aspergillus* sp., and *Penicillium* sp. are described as cellulase producers (Verma *et al.* 2010). Nevertheless, the lack of the complete

cellulase reduces the hydrolysis efficiency; for instance, the lack of β -glucosidase of *T. reesei* lowers the enzymatic hydrolysis. Researchers have focused on the isolation of microbes with a high production of the complete cellulase to achieve the efficient cellulose digestibility (Dashtban and Qin 2012).

Due to the recalcitrant structure of lignocelluloses, the utilization of steam explosion, acid, alkaline conditions, or ionic liquids (ILs) as a pretreatment have been a requirement. The ILs have been considered as an efficient pretreatment solvent (Fitzpatrick *et al.* 2010). This solvent can decompose the complex structure of lignocelluloses and reduce the crystallinity of cellulose to improve the subsequent enzymatic hydrolysis. The combination of ILs-pretreatment and cellulase hydrolysis has potential to greatly improve the bioconversion of lignocelluloses.

In this study, a series of microbes were isolated and identified with two dominant cellulase-producing strains. Fermentation factors, including carbon, nitrogen, initial pH, and fermentation time, were evaluated to improve the cellulase production. The NaCl stability and ILs stability of both microbes and cellulase were determined. The optimal pH and temperature of cellulase were studied for enzymatic hydrolysis. Enzymatic saccharification was investigated for corn stalk and ILs-pretreated corn stalk.

EXPERIMENTAL

Sample Collection

Soil samples were collected from the Greater Khingan Mountains (124.15951090674938E, 50.444505204846436N) in Heilongjiang province, China. Biomass was dried, ground, and passed through 40-mesh sieves. The chemicals pyridine (99%), 1,4-dichlorobutane (99%), 3-chloropropionic acid (99%), and potassium hexafluorophosphate (99%) were purchased from Aladdin Reagent Co., Ltd. (Shanghai, China).

Screening of Cellulase-producing Microbes

Each isolate was incubated on carboxyl methyl cellulose (CMC) medium including K_2HPO_4 (0.1%, weight per volume), NaCl (0.05%, weight per volume), $(NH_4)_2SO_4$ (0.2%, weight per volume), $MgSO_4$ (0.05%, weight per volume), sodium carboxymethyl cellulose (CMC-Na) (1.0%, weight per volume), and agar (1.0%, weight per volume) at 30 °C for 5 days (Wood *et al.* 1988). After that, the plates were stained with Congo-red solution (0.1%, weight per volume) and were discolored with 1 mol/L NaCl. One of the strains with a large hydrolysis zone was cultured in potato dextrose agar (PDA) medium including potato (0.2%, weight per volume), glucose (0.02%, weight per volume), and agar (1.0%, weight per volume) at 30 °C for 3 days (Xi *et al.* 2020).

A 1 cm agar piece was inoculated into potato dextrose (PD) medium including potato (0.2%, weight per volume), and glucose (0.02%, weight per volume). The solution was cultured at 30 °C and 150 rpm for 3 days. A total of 1 mL of the suspension (2%, volume per volume) was added into 100 mL of Hutchinson medium including $NaNO_3$ (0.25%, weight per volume), KH_2PO_4 (0.10%, weight per volume), $MgSO_4$ (0.03%, weight per volume), NaCl (0.01%, weight per volume), $CaCl_2$ (0.01%, weight per volume), $FeCl_3$ (0.001%, weight per volume) with corn stalk (1%, weight per volume), and peptone (0.2%, weight per volume). The medium was incubated at 30 °C and pH 7.0 and 150 rpm for 5

days. The solution was centrifuged at 4 °C with 10528 g for 10 min to provide the crude cellulase (Xi *et al.* 2020)

Determination of Cellulase Activity

The CMCase activity was measured with 1.5 mL of sodium carboxymethyl cellulose (1%, weight per volume) and 0.5 mL of the crude cellulase at 50 °C for 30 min. β -glucosidase activity was determined with 1.5 mL of salicin solution (0.5%, weight per volume) and 0.5 mL of the crude cellulase at 50 °C for 30 min. Filter paper (FPase) activity was measured with 50 mg of Whatman No. 1 filter paper (1.0 cm \times 6.0 cm) and 1.5 mL of sodium citrate buffer (pH 4.8) at 50 °C for 60 min. The reducing sugars were determined by the 3,5-dinitrosalicylic acid (DNS) method (Miller 1959; Papa *et al.* 2017). One unit (U) of the cellulase activity was defined as the amount of the enzyme that released 1 mmol of reducing sugar per min under the conditions indicated.

Molecular Identification

The DNA was extracted using a DNA kit based on the manufacturer's instructions (Omega BIO-TEC, Norcross, GA, USA). The universal primers ITS1 (5'-AGAAGTCGT-AACAAGGTTTCCGTAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGCTTAA-3') were used for the polymerase chain reaction. The products were sequenced at the Huada Gene Company (Beijing, China). The phylogenetic tree was constructed using the Molecular Evolutionary Genetics Analysis (MEGA) program (version 11, Mega Limited, Auckland, New Zealand).

Determination of Fermentation Factors

The carbon source (1%, w/v) and nitrogen source (0.2%, w/v) were determined at pH 7.0 for 5 days at 30 °C and 150 rpm with 2.0% (volume per volume) of inoculation of strains. The fermentation time was examined at pH 7.0 with corn stalk and peptone for the strain FLY4, and with corn stalk and beef extract for the strain FHH1. The initial pH was measured with corn stalk and peptone after 7 days for the strain FLY4. The initial pH was measured with corn stalk and beef extract after 9 days for the strain FHH1. The supernatant was obtained by centrifugation at 10,000 rpm for 10 min for the determination of cellulase activity.

Synthesis of Ionic Liquids

Ionic liquids were synthesized according to Han *et al.* (2019). Pyridine (1.0 mmol) and 1,4-dichlorobutane (1.0 mmol) were reacted at 60 °C for 4 days. The solid was purified by silica column chromatography (CH₂Cl₂/MeOH, 3/1) and was dried at 60 °C under vacuum for 4 h. After that, the product (1.0 mmol) was reacted with KPF₆ (1.0 mmol) in deionized water at room temperature for 3 h. The resulting product was dried in a vacuum for 12 h. Following that, the product (0.1 mmol) was reacted with pyridine (0.12 mmol) at 80 °C for 24 h. The resulting product was recrystallized with acetonitrile/ethyl acetate (1/10) and was dried in a vacuum at 60 °C for 4 h to give hexafluorophosphate [1-(1-pyridinium-yl)-butyl]-4-pyridinium chloride ([PF₆][(PYR)₄(PYR)][Cl]).

Pyridine (0.2 mol) was reacted with 3-chloropropionic acid (0.2 mol) at 70 °C for 24 h. After that, the product (0.1 mmol) was reacted with hexafluorophosphate (0.1 mmol) in 30 mL of deionized water at room temperature for 3 h. The resulting solid was filtered and

dried at 50 °C for 10 h to obtain [1-(2-carboxylpropyl)-pyridinium] hexafluorophosphate ((PYR)C₂COOH][PF₆]).

Determination of NaCl Stability and ILs Stability

The optimal pH and temperature for the crude cellulase were measured in citrate buffer at pH values in a range of 3.0 to 11.0 and temperatures in a range of 30 to 70 °C for 1 h. The stability of cellulase was measured in citrate buffer with the addition of NaCl or ILs at the optimal pH and temperature for different cellulase for 30 min.

Enzymatic Hydrolysis of Corn Stalk

Corn stalk (200 mg) was treated with 1.0 g of 1-(2-carboxylpropyl)-1-methylimidazolium hexafluorophosphate ([MIMC₂COOH][PF₆]) and 3.0 g of hexafluorophosphate [1-(1-pyridinium-yl-butyl)-4-methylimidazolium] chloride ([PF₆][PYRC₄MIM][Cl]) at 100 °C for 3 h, followed by the addition of 1.5 mL of deionized water for 3 h (Han *et al.* 2019). The residue was filtered and washed with water. Following that, the residue was dried in a vacuum at 50 °C for 6 h. The enzymatic reaction was performed with 100 mg of the original corn stalk or ILs-pretreated corn stalk and 5 mL of the crude enzyme in 15 mL of citrate buffer with the addition of 200 μ of NaN₃ (10 mg/μ L) for 24, 48, 72, and 96 h. The reducing sugar was measured by the DNS method (Miller 1959; Papa *et al.* 2017). The yields of reducing sugar were determined as the ratio of the mass of reducing sugar in the mass of corn stalk or ILs treated corn stalk that was subjected to enzymatic hydrolysis according to Eq. 1.

$$\text{Reducing sugar (\%)} = \frac{\text{Reducing sugar (mg)}}{\text{Corn stover or ILs treated corn stalk (mg)}} \times 100 \quad (1)$$

Statistical Analyses

Each experiment was performed in triplicate. The results are presented as mean values of three replicates with standard deviations (SD) shown as error bars. Statistical significance was determined by Student's t-test at significance levels indicated by asterisks as follows: *p ≤ 0.05, **p < 0.01, ***p < 0.001

RESULTS AND DISCUSSION

Identification of Cellulase-producing Microbes

A preliminary isolation of microbes was based on a Congo-red staining method. The isolates exhibiting clear hydrolytic zones were referred to as the potential cellulase-producing microbes. Two predominant strains exhibited high enzymatic activities, indicating their strong cellulase-producing capability.

The ITS sequence of DNA was used for the identifications of fungal strains. The isolate FHH1 was identified as *Penicillium janthinellum* FHH1, as it showed 99% similarity with *Penicillium janthinellum* (Fig. 1). The isolate FLY4 was identified as *Penicillium oxalicum* FLY4, as it showed 100% similarity with *Penicillium oxalicum*. Numbers of strains of *Penicillium* sp., such as *Penicillium janthinellum* EMS-UV-8, *Penicillium oxalicum* JU-A10-T, and *Penicillium oxalicum* GZ-2 were reported as good cellulase producers (Singhania *et al.* 2014; Liao *et al.* 2014; Yao *et al.* 2015; Agrawal *et*

al. 2017). The obtained nucleotide sequences were submitted to NCBI GeneBank under accession no. OK310849 for *P. janthinellum* FHH1 and OK310850 for *P. oxalicum* FLY4.

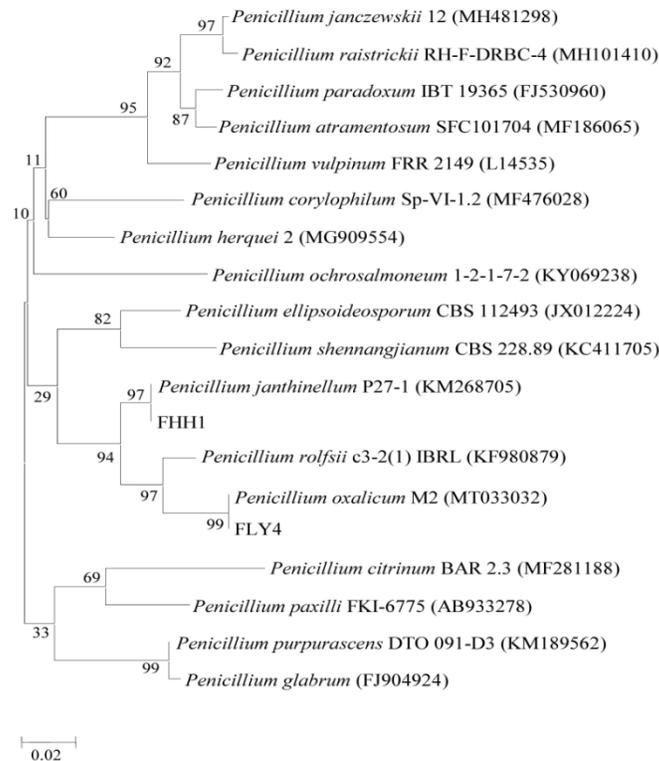


Fig. 1. The phylogenetic tree of the strains

Enzyme Activity in Different Carbon Sources

Carbon is a crucial nutrient for the growth and the reproduction of microbes. As shown in Fig. 2, corn stalk and rice straw were the effective carbon sources for *P. janthinellum* FHH1 and *P. oxalicum* FLY4. In particular, high productions of CMCase and β -glucosidase were observed with corn stalk as the carbon source. A high FPase production was obtained with rice straw as the carbon source. Nevertheless, low cellulase production was found with the utilization of CMC-Na and cellulose. As cellulase-producing activity of the strain could be inducible by cellulosic biomass, high cellulase productions were obtained for *P. janthinellum* FHH1 and *P. oxalicum* FLY4 using corn stalk as enzymatic substrates.

Agricultural wastes exhibit cellulolytic enzyme inducible properties. As reported, higher cellulase activities were obtained for *P. oxalicum* GZ-2 when lignocellulosic biomass was used instead of commercial cellulose (Liao *et al.* 2015). Similarly, congress grass was used as good carbon source for *Parthenium* sp. with high xylanase production (Dwivedi *et al.* 2009). Rice straw and wheat straw were found to be the most effective for FPase production (Liao *et al.* 2015). Corn cob and oat husk were the best inducers of xylanase for *Penicillium janthinellum* (Oliveira *et al.* 2006). Therefore, agricultural residue is more efficient than purified cellulose at inducing lignocellulolytic enzyme production (Liao *et al.* 2014). The use of agricultural wastes has both economic and environmental advantages.

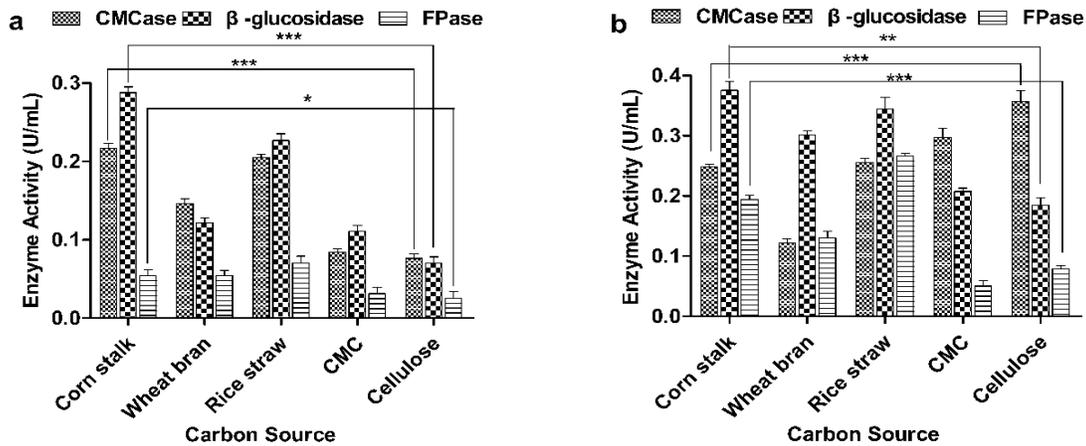


Fig. 2. Effect of carbon source on cellulase production: a) *P. janthinellum* FHH1 and b) *P. oxalicum* FLY4

Enzyme Activity in Different Nitrogen Sources

Nitrogen is a necessary nutrient for the growth of microbes, and it has an impact on their metabolic process. As presented in Fig. 3, high productions of CMCCase, β -glucosidase, and FPase were observed with beef extract as the nitrogen source for *P. janthinellum* FHH1. Low cellulase productions were found with the utilization of NH_4NO_3 and urea.

In addition, peptone and yeast extracts were the effective nitrogen sources for *P. oxalicum* FLY4. Specifically, high productions of β -glucosidase and FPase were observed with peptone as the nitrogen source. A high production of CMCCase was observed with yeast extract as the nitrogen source. Organic nitrogen on enzyme production was better than inorganic nitrogen for both strains. Therefore, beef extract was used for *P. janthinellum* FHH1 and peptone was used for *P. oxalicum* FLY4.

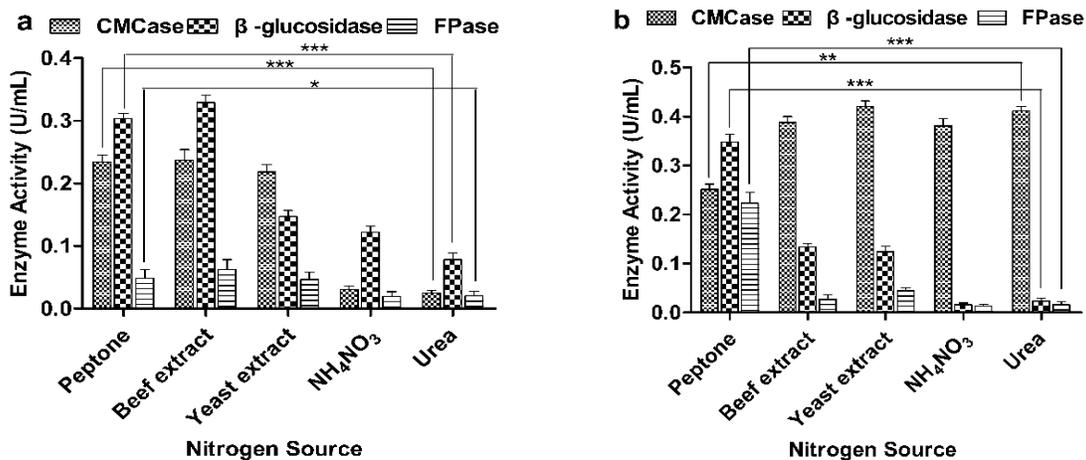


Fig. 3. Effect of nitrogen source on cellulase production: a) *P. janthinellum* FHH1 and b) *P. oxalicum* FLY4

Enzyme Activity after Different Fermentation Time

Cellulase production was improved with the increase of fermentation time (Fig. 4). A high yield of cellulase was obtained for *P. janthinellum* FHH1 after 9 days and for *P. oxalicum* FLY4 after 7 days. As the fermentation time increased, cellulase production was decreased due to the reduction and the depletion of nutrients. Moreover, the higher ratio of β -glucosidase and FPase of *P. janthinellum* FHH1 and *P. oxalicum* FLY4 was capable of improving the inhibition of enzymatic hydrolysis due to cellobiose accumulation.

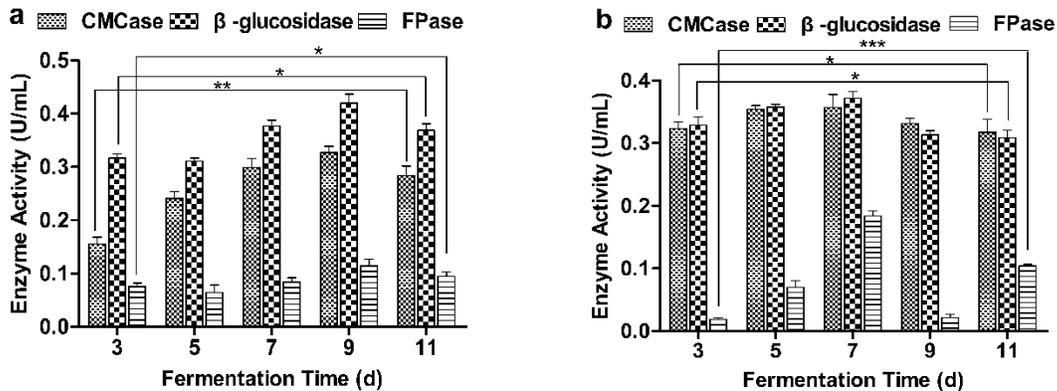


Fig. 4. Effect of fermentation time on cellulase production. a) *P. janthinellum* FHH1 and b) *P. oxalicum* FLY4

Enzyme Activity at Different Initial pH

Filamentous fungi have good growth over a broad range from pH 2 to 9 with an optimal range from 3.8 to 6.0 (Gowthaman *et al.* 2001). *P. janthinellum* FHH1 preferred acidic pH conditions (3.0 to 5.0). Particularly, this strain possessed a high cellulase production at pH 9.0. In addition, a broad pH range from 3.0 to 11.0 was applicable for *P. oxalicum* FLY4 with pH 5.0 being optimal (Fig. 5). High production of CMCase and β -glucosidase was obtained at pH 7.0 and pH 5.0, respectively.

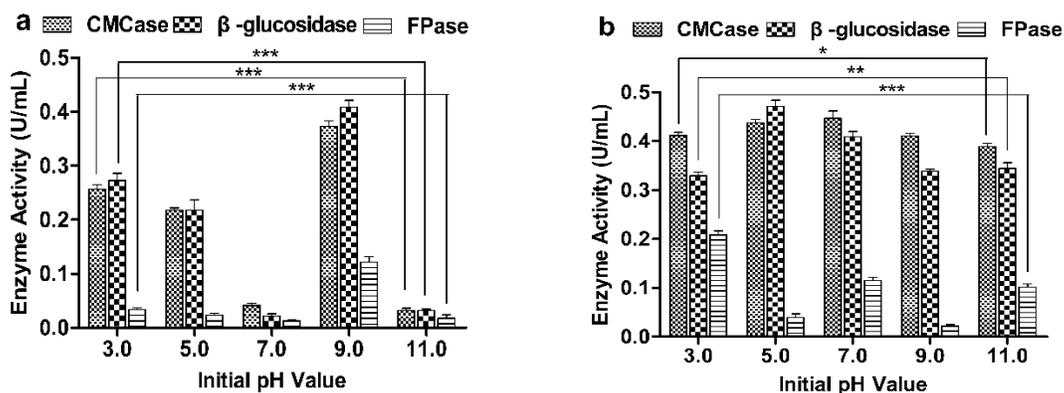


Fig. 5. Effect of initial pH value on cellulase production: a) *P. janthinellum* FHH1 and b) *P. oxalicum* FLY4

pH Value on Cellulase Activity during Hydrolysis

The pH value influences the stability of cellulase during the hydrolysis. Inappropriate pH may cause the destruction of the spatial structure and the dissociation

state of the enzyme (Adsul *et al.* 2007). A pH value between 3.0 and 6.0 was applicable for cellulase of *P. janthinellum* FHH1 (Fig. 6). High activities of CMCase and β -glucosidase were obtained at pH 4.0. A high FPase activity was observed at pH 5.0. Meanwhile, a pH value from 4.0 to 6.0 was applicable for *P. oxalicum* FLY4 with pH 4.0 being optimal for CMCase, β -glucosidase, and FPase. The CMCase and β -glucosidase of *P. oxalicum* GZ-2 showed remarkable stability in the pH range 4.0 to 9.0 and pH 3.0 to 7.0 with 80% of the maximum activity (Liao *et al.* 2015). Optimum pH for activity of the crude enzyme of *Penicillium oxalicum* IODBF-5 were pH 5 (Saini *et al.* 2015). Thus, an acidic condition was preferred for *P. janthinellum* FHH1 and *P. oxalicum* FLY4 in this study.

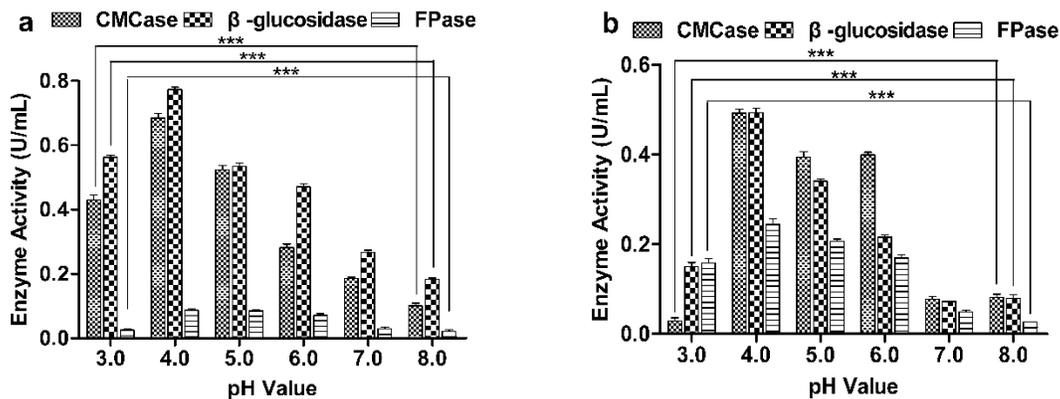


Fig. 6. Effect of pH value on cellulase activity: a: *P. janthinellum* FHH1; b: *P. oxalicum* FLY4

Hydrolysis Temperature on Cellulase Activity

A low temperature may decrease the rate of enzymatic reaction, while a high temperature may denature the protein leading to the loss of enzyme activity (Larsson *et al.* 2001). As the temperature was increased, the activities of β -glucosidase and CMCase for *P. janthinellum* FHH1 were improved with the maximum activity at 60 and 50 °C, respectively (Fig. 7).

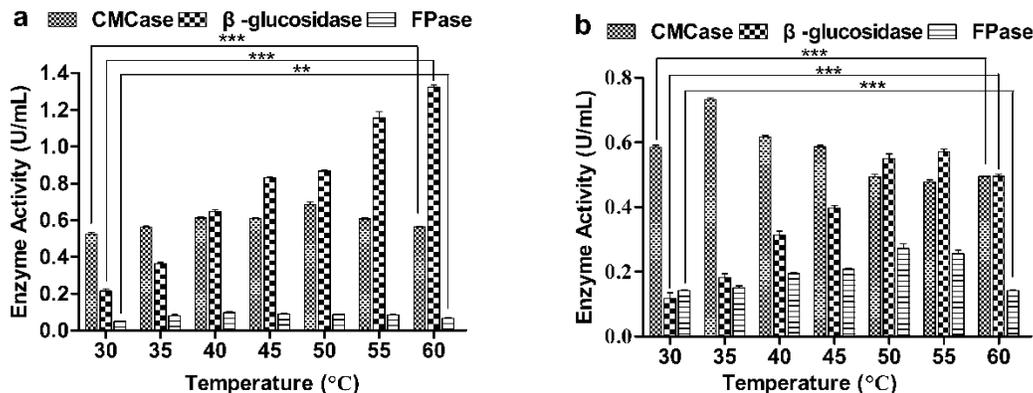


Fig. 7. Effect of hydrolysis temperature on cellulase activity: a) *P. janthinellum* FHH1 and b) *P. oxalicum* FLY4

No obvious reduction of FPase activity was found with the temperature variation. As reported, the optimal temperatures for CMCase and β -glucosidase activity were at 50

and 60 °C, respectively (Liao *et al.* 2015). The optimum temperature for activity of the crude enzyme of *Penicillium oxalicum* IOBF-5 were 50 °C (Saini *et al.* 2015). A high temperature was found for CMCase and β -glucosidase of *P. janthinellum* FHH1. Meanwhile, when the temperature was increased, the activities of β -glucosidase and FPase for *P. oxalicum* FLY4 were improved with the maximum activity at 55 and 50 °C, respectively. However, the optimal temperature was found at 35 °C for CMCase of *P. oxalicum* FLY4. Thus, CMCase activity was reduced with the increase of the temperature.

NaCl Stability of Cellulase

When NaCl concentration was increased from 2.5% to 7.5% (weight per volume), NaCl stability of CMCase was improved for *P. janthinellum* FHH1 (Fig. 8). No obvious reduction of FPase stability was found. Nevertheless, the stability of β -glucosidase was reduced with the increase of NaCl concentration.

Moreover, an improved CMCase activity was achieved for *P. oxalicum* FLY4 in a range from 25% to 35% (w/v) of NaCl concentration with the maximum CMCase activity at 15% (w/v). NaCl precipitated out of the solution at 40% (w/v). A slight reduction of the stability of β -glucosidase and FPase was found with the increase of NaCl concentration. Therefore, a high NaCl stability was achieved for *P. oxalicum* FLY4.

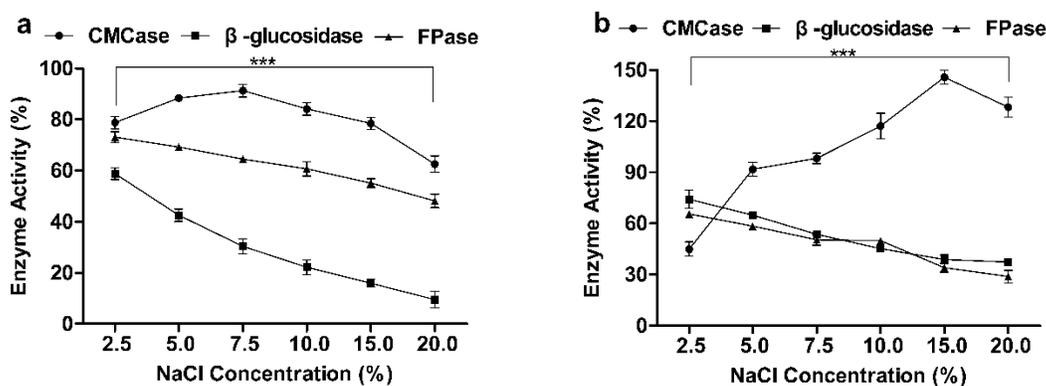


Fig. 8. NaCl-stability of cellulase. a: *P. janthinellum* FHH1; b: *P. oxalicum* FLY4

ILs Stability of Cellulase

A higher ILs stability was achieved with cellulase obtained from *P. oxalicum* FLY4 than that of *P. janthinellum* FHH1 (Fig. 9). A total of 66% and 63% of the CMCase activity was retained for *P. oxalicum* FLY4 with the addition of 2.5% (w/v) of [(PYR)₂COOH][PF₆], and [PF₆][(PYR)₄(PYR)][Cl], respectively. A total of 57% and 51% of the CMCase activity was retained for *P. janthinellum* FHH1 with [(PYR)₂COOH][PF₆], and [PF₆][(PYR)₄(PYR)][Cl], respectively. The stability of CMCase was higher than β -glucosidase. It was reported that β -glucosidase was more stable than CMCase for *P. janthinellum* mutants, as CMCase showed 90% of its initial activity in 10% of 1-butyl-3-methylimidazolium chloride ([bmim]Cl) and β -glucosidase retained 85% of its original activity in 30% of ionic liquid (Adsul *et al.* 2009). In addition, CMCase that was obtained from *P. oxalicum* FLY4 retained 86% of the original activity with [EMIM]CH₃COOH. A higher stability was found with [EMIM]CH₃COOH for both strains.

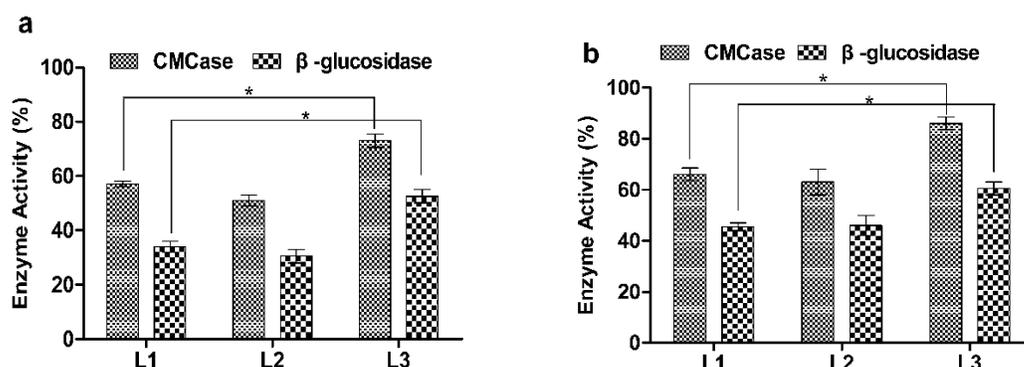


Fig. 9. ILs stability of cellulase: a) *P. janthinellum* FHH1 and b) *P. oxalicum* FLY4; L1: [(PYR)₂C₂COOH][PF₆], L2: [PF₆][(PYR)₄(PYR)][Cl] and L3: [EMIM]CH₃COOH

Enzymatic Hydrolysis of Corn Stalk

The enzymatic reaction was performed with 100 mg of the original corn stalk and 5 mL of the crude cellulase for 24, 48, 72, and 96 h at pH 4.0 for *P. janthinellum* FHH1. The temperature conditions varied at 60 °C for FHH1 and 35 °C for *P. oxalicum* FLY4. As the hydrolysis time was increased from 24 to 96 h, the reducing sugars yield was improved for both of strains. After 96 h, the reducing sugars yield was 18% for *P. janthinellum* FHH1 and 33.75% for *P. oxalicum* FLY4. A higher performance of cellulase of *P. oxalicum* FLY4 was achieved than that of *P. janthinellum* FHH1 (Fig. 10). Compared with acid pretreated and steam exploded wheat straw, the untreated wheat straw could be a good carbon source for the production of quality cellulolytic enzymes by *P. janthinellum* EMS-UV-8 mutant (Sharma *et al.* 2015). This is different with *P. janthinellum* FHH1 and *P. oxalicum* FLY4, because high hydrolysis efficiency was observed with ILs-pretreated corn stalk.

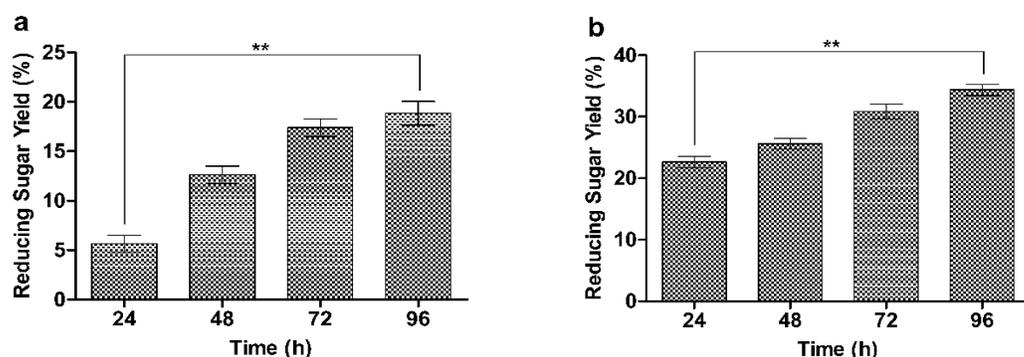


Fig. 10. Enzymatic hydrolysis of corn stalk: a) *P. janthinellum* FHH1 and b) *P. oxalicum* FLY4

Enzymatic Hydrolysis of ILs-pretreated Corn Stalk

During the treatment by the mixture of [PF₆][(PYR)₄(PYR)][Cl] and [(PYR)₂C₂COOH][PF₆] at 100 °C, a high reducing sugar yield for corn stalk was achieved at 18.80% (Han *et al.* 2019). The residue was obtained at 68.75% after ILs treatment. The enzymatic reaction was conducted with 100 mg of ILs-pretreated corn stalk and 5 mL of the crude cellulase for 24, 48, 72, and 96 h for *P. janthinellum* FHH1 at pH 4.0 and 60 °C and for *P. oxalicum* FLY4 at pH 4.0 and 35 °C. The reducing sugar yield increased with the increase of hydrolysis time (Fig. 11). After 96 h, the reducing sugar yield was 30.93%

for *P. janthinellum* FHH1. A high yield was achieved of 52.37% for *P. oxalicum* FLY4 after 72 h. Meanwhile, the yield of the reducing sugar was 42.21% for [EMIM]CH₃COOH-pretreated corn stalk. Hydrolysis of acid or alkali-pretreated rice straw using cellulase enzyme preparations from *P. janthinellum* and *T. reesei* released 37% and 43% glucose, respectively (Sreeja-Raju *et al.* 2020). A crude enzyme solution of *P. oxalicum* Z1-3 was able to hydrolyze 92.5% of KOH-pretreated sugarcane bagasse to glucose within 96 h (Jing *et al.* 2015). A crude enzyme solution of *Penicillium oxalicum* EU2106 was able to hydrolyze 94.3 ± 1.5% of NaOH-H₂O₂-pretreated cassava residue to glucose under solid-state fermentation for 96 h (Su *et al.* 2017). In the present work, a high production of reducing sugar was achieved with the hydrolysis of [PF₆][(PYR)C₄(PYR)][Cl] / [(PYR)C₂COOH][PF₆]-pretreated corn stalk by cellulase obtained from *P. oxalicum* FLY4.

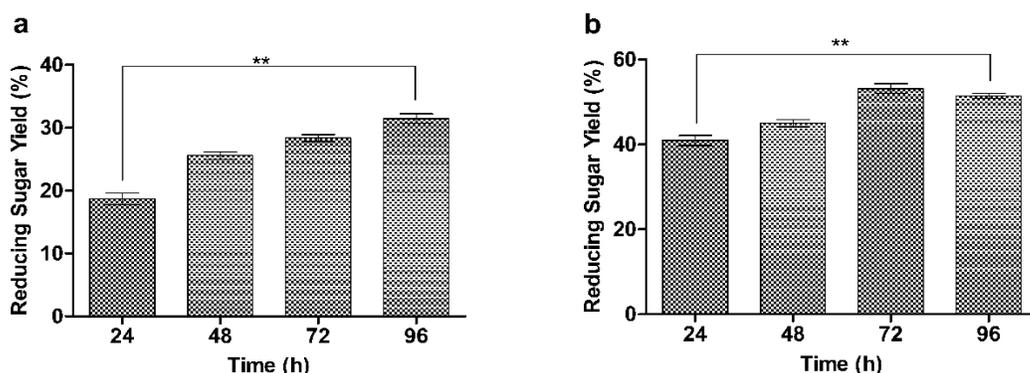


Fig. 11. Enzymatic hydrolysis of ILs-pretreated corn stalk: a) *P. janthinellum* FHH1 and b) *P. oxalicum* FLY4

Herein, cellulases from *P. janthinellum* FHH1 and *P. oxalicum* FLY4 have been successfully used for saccharification of corn stalk. Both of the strains from *Penicillium* species exhibit remarkable saccharification performance for supplementation of β -glucosidase-deficient *Trichoderma* cellulases (Gusakov and Sinitsyn 2012). Agricultural wastes act as non-food resource to save costs of the enzyme production process. In addition, the acidic group (COOH⁻) of ILs assists the hydrolysis of hemicellulose and cellulose of corn stalk. The anion (Cl⁻) of ionic liquids made it more effective at disrupting inter- and intra-molecular hydrogen bonding in lignocelluloses. Thus, the effective ILs-treatment of lignocelluloses contributed to achieve the high enzymatic digestibility. These cellulases from *P. janthinellum* FHH1 and *P. oxalicum* FLY4 are potential candidates to be utilized in hydrolyzing the lignocellulosic biomass.

CONCLUSIONS

1. The fungal strains with high cellulase productions were identified as *P. janthinellum* FHH1 and *P. oxalicum* FLY4. Corn stalk was considered as the optimal carbon source for both strains. Beef extract and peptone were the optimal nitrogen source for *P. janthinellum* FHH1 and *P. oxalicum* FLY4, respectively.
2. High cellulase activities were obtained in a pH range of 4.0 to 6.0 and a temperature between 55 and 60 °C during hydrolysis. A higher NaCl stability and ILs stability was observed for cellulase obtained from *P. oxalicum* FLY4 than *P. janthinellum* FHH1.

3. High reducing sugar yields were achieved by enzymatic hydrolysis of ILs-pretreated corn stalk. A combination of ILs pretreatment and cellulase saccharification will have a successful utilization for the degradation of lignocellulosic biomass into value-added products.

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