

Effect of Glucose on Endo-xylanase and β -xylosidase Production by Fungi Isolated in Indonesia

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Abstract

Xylanases are widely produced by fungi, and the production of polysaccharide-degrading enzymes, in general, are usually subjected to carbon catabolite repression. In this work, the ability of several Indonesian indigenous fungi to produce endo-xylanase and β -xylosidase and their responses to glucose as a repressor were determined. Ten fungi were grown in a liquid medium supplemented with glucose as the repressor (0, 1%, 3%, and 5%), and the endo-xylanase and β -xylosidase productions were assayed. *Aspergillus aculeatus* FIG1 and *A. oryzae* KKB4 produced 3.85 and 0.70 U/mL of endo-xylanase, respectively, compared with other strains (0.22 U/mL or less). *Trichoderma asperellum* PK1J2, *T. virens* MLT2J2, *A. aculeatus* FIG1, *T. asperellum* MLT5J1, *A. oryzae* KKB4, and *T. asperellum* MLT3J2 produced 0.021–0.065 U/mL of β -xylosidase, whereas the other strains produced 0.013 U/mL or less of β -xylosidase. Adding 1% glucose to the growth medium can partially repress endo-xylanase production in *A. aculeatus* FIG1, *T. asperellum* PK1J2, and *T. virens* MLT4J1 and completely repress other strains. By adding 1% glucose, strains FIG1, PK1J2, and MLT4J1 suffered almost complete repression of β -xylosidase production, although such strains exhibited partial repression of endo-xylanase production. β -Xylosidase produced by the other strains showed complete repression by adding 1% glucose, except for *A. aculeatus* FIG1, *A. tamarii* FNCC 6151, and *T. asperellum* MLT1J1, which showed partial repression. Therefore, adding 3% glucose to the growth medium can result in complete repression of endo-xylanase and β -xylosidase productions in all strains examined.

Keywords: Endo-xylanase, β -xylosidase, carbon catabolite repression, glucose

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INTRODUCTION

Xylanases, including hydrolyze xylan to xylose and xylooligosaccharides, are produced by fungi, *Actinomycetes*, and bacteria. Xylanases are widely used in the food industry, particularly in the production of xylose and xylooligosaccharides, purification of juice and wine, and improvement of dough quality.¹⁻³ Hydrolysis of xylan to xylose and xylooligosaccharides can make juice and wine clearer, reduce dough adhesiveness, and increase dough consistency.^{4,5}

Filamentous fungi can produce more xylanases than yeast, *Actinomycetes*, and bacteria.² Filamentous fungi are also more suitable to be used in solid-state fermentation, which can increase the production of enzymes.⁶ Different species of filamentous fungi produce xylanase, such as *Aspergillus* sp., *Trichoderma* sp., *Penicillium* sp., *Fusarium* sp., and *Pseudomonas* sp.⁷ Based on previous reports, fungi are good producers of lignocellulolytic enzymes. From an industrial point of view, fungi produce extracellular enzymes that are necessary to hydrolyze lignocellulose materials or several enzymes that are necessary to debranch substituted polysaccharides.⁸ The yield of fungi is also higher than that of yeast and bacteria. Fungi from soil or plants show potential application for xylanase production in industries because they have adapted to hemicellulosic materials.

Although many fungi can produce enzymes at a different level, fungi with different strains could produce different enzymatic activities. *A. tubingensis* KRCF 700 is the highest cellulase and xylanase producer in CMC and xylan medium among other fungi based on the screening of 16 fungi.⁹ *Trichoderma viride* Fd18

shows the highest specific xylanase activity of 0.75 U/mL protein based on microbial screening among soil, decaying wood logs, and sawdust.¹⁰ *Pseudodaphnella stipata* SC 04 and *Botryosphaeria* sp. AM 01 are good producers of xylanase and β -xylosidase with enzymatic activity of 694.33 and 4.87 U/g.¹¹ The selection of a particular strain to obtain a high enzyme yield must be achieved. Therefore, screening indigenous fungi is important to determine efficient fungi that can produce endo-xylanase and β -xylosidase.

However, the use of fungi to produce hydrolytic enzymes is hindered by the presence of carbon catabolite repression (CCR), which is a mechanism that regulates gene expression by using a compound that can be easily metabolized as a carbon source. Carbon catabolite repression switches off certain enzymes required to utilize less favored carbon sources when a more readily available carbon source is present in the medium.¹² Other research has reported that the presence of glucose in the medium induces the growth of fungi, which causes high secretion of enzymes. Therefore, elevated glucose concentration increases amylase production.¹³ In addition, the presence of glucose, sucrose, and galacturonic acid in the medium containing pectin does not reduce pectinase activity.¹⁴ However, the addition of glucose to the liquid medium increases tannase activity at concentrations of 6.25–25 g/L but decreases the enzymatic activity when glucose concentration reaches 50 g/L.¹⁵ Based on the different responses to the presence of glucose, the effect of glucose on endo-xylanase and β -xylosidase in our isolates must be examined.

Table 1. Fungi used in this study and its origin

Fungi	Origin	Accession Number
<i>Aspergillus aculeatus</i> FIG1	Cacao pod, Gunung Kidul	OK336358
<i>Aspergillus oryzae</i> KKB4	Koji, Kebumen	OK336357
<i>Aspergillus tamarii</i> FNCC 6151	Koji, Bantul	OK336356
<i>Penicillium citrinum</i> G2J2	Oil palm empty fruit bunch, Garut	OK336360
<i>Trichoderma asperellum</i> PK1J2	Oil palm empty fruit bunch, Pekanbaru	OK336355
<i>Trichoderma asperellum</i> MLT1J1	Coconut husk, Central Maluku	OK336350
<i>Trichoderma virens</i> MLT2J2	Coconut husk, Central Maluku	OK336351
<i>Trichoderma asperellum</i> MLT3J2	Coconut stalk, Central Maluku	OK336352
<i>Trichoderma virens</i> MLT4J1	Coconut rotten wood, Central Maluku	OK336353
<i>Trichoderma asperellum</i> MLT5J1	Soil, Central Maluku	OK336354

In this study, fungi isolated from various sources in Indonesia were examined for their ability to produce endo-xylanase and β -xylosidase, and the presence of CCR was observed by adding glucose as a readily metabolized carbon source for the production of enzymes.

MATERIALS AND METHODS

Microorganisms

The fungal strains used in this study were provided by the Biotechnology Laboratory at the Department of Food and Agricultural Product Technology, Gadjah Mada University. The strains were isolated from various sources in Indonesia. Such strains are shown in Table 1. The strains were kept on a potato dextrose agar slant at 4 °C.

Screening of xylanase-producing fungi in a solid medium

The fungi were grown at 30 °C for 4 days in a solid medium as described by Mandels and Reese¹⁶, which has been modified by Chand et al.¹⁷ The medium consisted of urea (0.3 g/L), $(\text{NH}_4)_2\text{SO}_4$ (1.4 g/L), KH_2PO_4 (2.0 g/L), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.4 g/L), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.3 g/L), peptone (1.0 g/L), tween (80 0.2 g/L), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (5.0 mg/L), $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$ (1.6 mg/L), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (1.4 mg/L), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (20.0 mg/L), agar (17.5 g/L), and 0.1% Triton X-100. The carbon source used was xylan at 10.0 g/L. The pH of the medium was adjusted to 5.0. Then, the medium plates were stained using 0.1% of Congo red and incubated for 20 min. After washing with 1 M NaCl, the diameter of clear zones formed was measured. The xylanolytic activity of the strains was estimated by using a clear zone ratio.^{6,17}

Clear zone ratio = (Clear zone diameter - Colony diameter) / (Colony diameter)

Xylanase production in liquid culture

The fungi were grown in a liquid medium at 30 °C for 4 days. The medium used was identical to the solid medium except for agar and Triton X-100, which were omitted from the medium. The broth was centrifuged at 10,000 rpm at 4 °C for 10 min. The cell-free supernatant was used as a crude enzyme, and its endo-xylanase and β -xylosidase activities were analyzed.

Effect of glucose on endo-xylanase and β -xylosidase production

The fungi were grown in a liquid medium supplemented with various amounts of glucose

(1%, 3%, and 5%) at 30 °C for 4 days. After incubation, the endo-xylanase and β -xylosidase activities in the cell-free supernatant were analyzed.

Analysis of endo-xylanase activity

Analysis of endo-xylanase activity was conducted using the XylX6 method from Megazyme Ltd., Ireland, according to the manufacturer's instructions. The substrate used in the assay was xylohexoside, which was blocked at the non-reducing end, and a nitrophenyl moiety was added at the other end. The action of endoglucanase generated a non-blocked xylooligosaccharide that was rapidly hydrolyzed by β -xylosidase, which was included in the substrate. The 4-nitrophenol formed was measured at 400 nm. One unit of endo-xylanase was defined as the amount of enzyme required to release 1 μmol of 4-nitrophenol in 1 min.

Analysis of β -xylosidase activity

The β -xylosidase activity was measured following the methods of Kundu et al.¹⁸ A sample containing β -xylosidase was added to p-nitrophenyl β -D-xylopyranoside (Megazyme) as a substrate and incubated for 15 min at 55 °C. The reaction was ended by adding sodium carbonate as a stopping reagent, and the p-nitrophenol formed at 405 nm was measured. One unit β -xylosidase activity was defined as the amount of enzyme required to release 1 μmol of 4-nitrophenol in 1 min under the defined assay condition.¹⁸

Statistical analysis

The statistically treated data were presented as the arithmetical mean values and their standard errors. Data were analyzed using one-way analysis of variance, and differences among means were compared using the Duncan test ($\alpha = 0.05$).

RESULTS AND DISCUSSION

Xylanase production

The fungal strains that produce xylanases were initially screened by observing the clear zones surrounding the colonies in the plate medium after staining with Congo red. All strains examined showed clear zones surrounding the colonies, which indicated that the strains produced xylanases. The size of the clear zone was expressed as the ratio among different clear zones, colony diameters, and colony diameter. A clear zone ratio

equals to zero indicated no clear zone. The clear zone ratio of each strain varied from 0.36 to 1.02 (Table 2). Among the strains, FIG1, KKB4, FNCC 6151, G2J2, and MLT4J1 had the highest clear zone ratios.

All strains examined produced endo-xylanase and β -xylosidase in a liquid medium (Table 3). Strain FIG1 produced 3.85 U/mL of endo-xylanase after 4 days. This amount was considerably higher than that of endo-xylanase produced by the other strains, which was between 0.02 and 0.70 U/mL. Strain KKB4 produced a moderate amount of endo-xylanase, which was 0.70 U/mL, and the rest of the strains produced 0.22 U/mL or less of endo-xylanase. To date, no report on endo-xylanase production by

microorganisms has been found. Most reports on xylanase production analyzed the enzyme as total xylanase. Researchers used xylan as a substrate and measured the reducing sugars as the product of hydrolysis. Xylanase production measured as total xylanases was reported by workers. *A. niger* NBRC 31125, *A. tubingensis* KRCF 700, *Grammothele fuligo* WD 844, *Perenniporia fraxinea* WD 1518, *Phanerochaete chrysosporium* WD 1416, *Pycnoporus coccineus* WD 2263, *T. harzianum* KRCF 131, *T. reesei* NBRC 31329, and *Trichoderma* sp. (four strains) produced xylanase between 0.87 and 7.00 U/mL.⁹ Another study reported that 50 fungal strains were screened for xylanase production and found that the xylanase production of the top 10 isolates was between 6.43 and 38.90 U/mL.¹⁹

Among the 10 strains examined, the strain that produced the highest amount of endo-xylanase did not produce the highest amount of β -xylosidase. Strain FIG1 was the highest producer of endo-xylanase, whereas strain PK1J2 was the highest producer of β -xylosidase, which produced 0.065 U/mL of β -xylosidase. Strains MLT2J2, FIG1, MLT5J1, KKB4, and MLT3J2 produced β -xylosidase between 0.021 and 0.036 U/mL, and the rest of the strains produced only 0.002 to 0.013 U/mL of β -xylosidase. Strains FIG1 and KKB4 were considered as high producers of endo-xylanase and β -xylosidase; they produced 3.85 and 0.70 U/mL of endo-xylanase, respectively, and 0.031 and 0.021 of β -xylosidase, respectively. However, the other strains produced a relatively high amount of endo-xylanase but a low amount of β -xylosidase or vice

Table 2. Clear zone ratio of indigenous strains in xylan agar incubated for 4 days

Fungi	Clear zone ratio
<i>Aspergillus aculeatus</i> FIG1	0.96±0.31 ^{bcd}
<i>Aspergillus oryzae</i> KKB4	0.75±0.35 ^{bcd}
<i>Aspergillus tamarii</i> FNCC 6151	0.86±0.20 ^{bcd}
<i>Penicillium citrinum</i> G2J2	0.83±0.07 ^{bc}
<i>Trichoderma asperellum</i> PK1J2	0.59±0.09 ^{bc}
<i>Trichoderma asperellum</i> MLT1J1	0.61±0.00 ^{bc}
<i>Trichoderma virens</i> MLT2J2	0.65±0.03 ^{bcd}
<i>Trichoderma asperellum</i> MLT3J2	0.36±0.23 ^{ab}
<i>Trichoderma virens</i> MLT4J1	1.02±0.39 ^c
<i>Trichoderma asperellum</i> MLT5J1	0.53±0.19 ^{abc}

*Each value is a mean of two replicates; \pm stands for standard deviation among replicates; alphabet means followed by different letters within a column differ significantly at $\alpha \leq 0.05$

Table 3. Production of endo-xylanase and β -xylosidase of indigenous strains in a xylan medium incubated for 4 days

Fungi	Endo-xylanase (U/mL)	β -xylosidase (U/mL)
<i>Aspergillus aculeatus</i> FIG1	3.85±1.06 ^a	0.031±0.001 ^a
<i>Aspergillus oryzae</i> KKB4	0.70±0.06 ^b	0.021±0.001 ^d
<i>Aspergillus tamarii</i> FNCC 6151	0.22±0.02 ^b	0.002±0.001 ^e
<i>Penicillium citrinum</i> G2J2	0.11±0.02 ^b	0.005±0.002 ^e
<i>Trichoderma asperellum</i> PK1J2	0.06±0.00 ^b	0.065±0.006 ^b
<i>Trichoderma asperellum</i> MLT1J1	0.10±0.00 ^b	0.013±0.004 ^d
<i>Trichoderma virens</i> MLT2J2	0.16±0.02 ^b	0.036±0.001 ^c
<i>Trichoderma asperellum</i> MLT3J2	0.11±0.03 ^b	0.021±0.003 ^d
<i>Trichoderma virens</i> MLT4J1	0.02±0.00 ^b	0.004±0.002 ^e
<i>Trichoderma asperellum</i> MLT5J1	0.16±0.02 ^b	0.023±0.000 ^d

*Each value is a mean of two replicates; \pm stands for standard deviation among replicates; alphabet means followed by different letters within a column differ significantly at $\alpha \leq 0.05$

versa. Previous studies on β -xylosidase reported that the β -xylosidase production of four species of *Aspergillus* was between 0.017 and 0.149 U/mL.²⁰ Other works reported that 12 species of *Aspergillus*, *Beauveria*, *Mucor*, *Paecilomyces*, and *Penicillium* were examined for their ability to produce β -xylosidase and showed that such strains produced 0.030–0.723 of β -xylosidase.²¹ Another research reported that *T. asperellum* produced 0.19 U/mL of β -xylosidase.²²

All fungi examined the produced endo-xylanase, which was considerably higher than β -xylosidase. All previous works reported similar results. The strains produced more endo-xylanase than β -xylosidase. The hydrolysis of xylan to xylose might require more endo-enzyme that cleavages the polymer formed inside than β -xylosidase, which cut the xylooligosaccharide produced by hydrolyzing endo-xylanase to xylose.

The strains that provided high clear zone ratios did not produce a high amount of xylanase in the liquid medium (Tables 2 and 3). Among the strains that produced high clear zone ratios, *T. virens* MLT4J1, *A. tamaritii* FNCC 6151, and *P. citrinum* G2J2 produced only 0.02, 0.22, and 0.11 U/mL of endo-xylanase, respectively, and no β -xylosidase. However, *A. aculeatus* FIG1 and *A. oryzae* KKB4 were considered as high producers of xylanases in solid and liquid media. In contrast, strains FIG1 and KKB4 produced 3.85 and 0.70 U/mL of endo-xylanase, respectively, and 0.03 and 0.02 U/mL of β -xylosidase, respectively. The other strains examined produced significantly less endo-xylanase and β -xylosidase than strains FIG1 and KKB4 in a liquid medium.

Effect of glucose on the production of endo-xylanase and β -xylosidase

Xylanases are subjected to CCR, where xylose, glucose, or other sugar can repress the

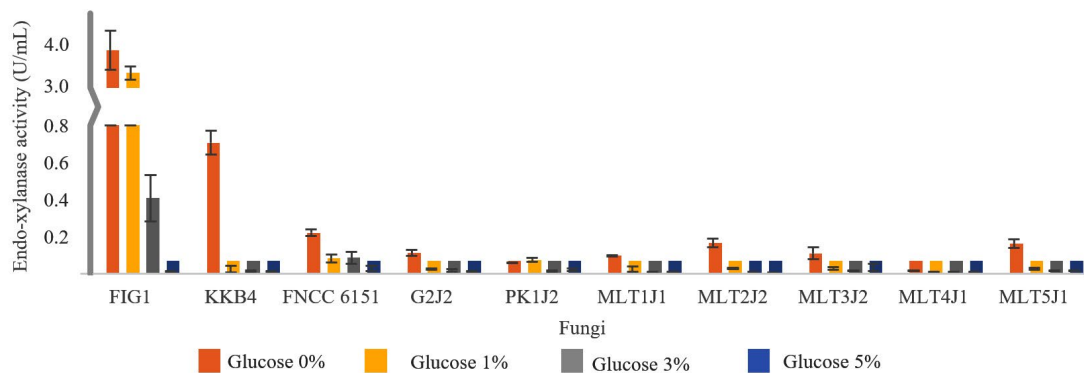


Fig. 1. Endo-xylanase production after the strains were grown in a medium supplemented with various amounts of glucose for 4 days.

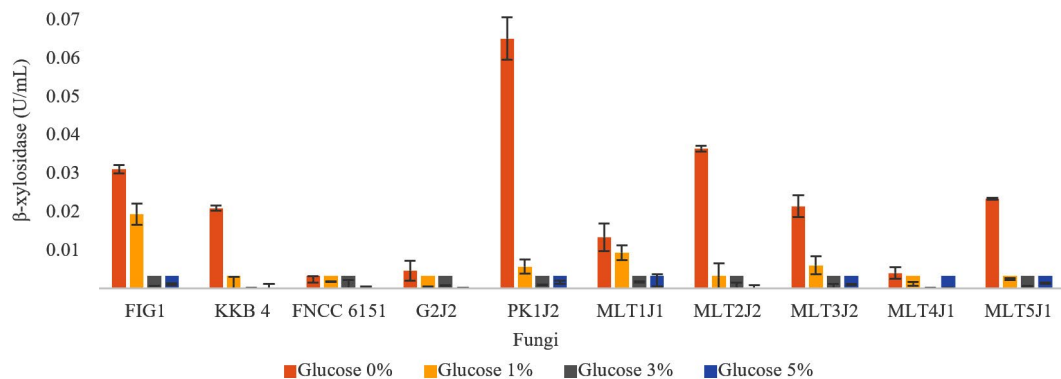


Fig. 2. β -Xylosidase production after the strains were grown in a medium supplemented with various amounts of glucose for 4 days.

synthesis of xylanases. In this study, the production of endo-xylanase and β -xylosidase was examined to determine whether it was affected by the presence of glucose as a readily metabolized sugar in the medium.

As shown in Fig. 1, the addition of glucose significantly decreased the production of endo-xylanase. The addition of 1% glucose to the growth medium decreased the endo-xylanase production by less than 50% in strains FIG1, PK1J2, and MLT4J1 and by more than 95% in strain KKB4. Meanwhile, strains FNCC 6151, G2J2, MLT1J1, MLT2J2, MLT3J2, and MLT5J1 decreased the endo-xylanase production from 63% to 84% when 1% glucose was added in the medium. The degree of repression of the production of endo-xylanase after adding 3% glucose was not significantly different from that after adding 5% glucose. In general, the production of endo-xylanase in the presence of 3% glucose or more in all strains was low.

The production of β -xylosidase in all strains was repressed when the growth medium was supplemented with glucose. In strains FIG1, FNCC 6151, and MLT1J1, the addition of 1% glucose to the growth medium decreased the β -xylosidase production by less than 50%. By contrast, the β -xylosidase production in strains KKB4, G2J2, PK1J2, MLT2J2, and MLT5J1 decreased by 90% or more when 1% glucose was added. Meanwhile, the addition of 1% glucose to the medium decreased β -xylosidase production by 72%–89%

in strains MLT3J2 and MLT4J1. The addition of 3% glucose or more to the growth medium slightly decreased the β -xylosidase production in all strains examined, as shown in Fig. 2.

The presence of CCR in endo-xylanase and β -xylosidase production was observed in *A. nidulans*, *A. versicolor*, and *Streptomyces*.²³⁻²⁵ The presence of 1% glucose in the medium drastically decreased β -xylosidase production by 91.82% in *A. versicolor* after incubation for 72 h.²⁴ A similar result showed that the addition of 1% glucose to the culture medium of *A. nidulans* slightly inhibited the production of xylanase; however, the activity of xylanase started to be detected after 24 h when glucose concentration dropped to almost zero. Therefore, *A. nidulans* initially consumed glucose as a carbon source and then the products of xylan hydrolysis when the amount of glucose in the medium was closed to zero.²³ The addition of glucose, glycerol, and succinic acid in the culture medium individually at 1% increased the number of carbon sources that strongly repressed endo-xylanase and β -xylosidase production in *Streptomyces*. The addition of 1% xylose to the culture medium reduced the production of endo-xylanase and β -xylosidase in *Streptomyces* by 81.7% and 65.1%, respectively.²⁵

A. aculeatus FIG1 could be used for endo-xylanase and β -xylosidase production. Endo-xylanase is an important enzyme in the production of xylooligosaccharides.^{26,27} and it is

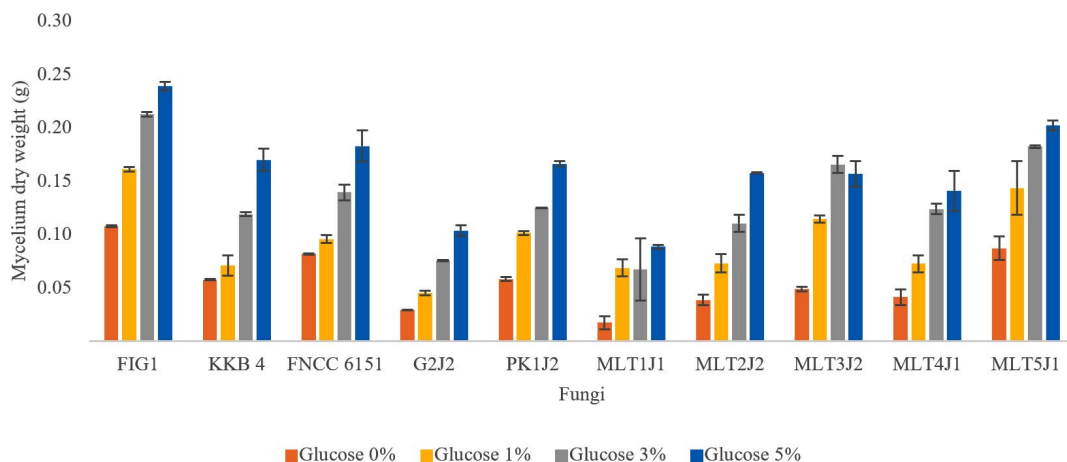


Fig. 3. Biomass produced after the strains were grown in a medium supplemented with various amounts of glucose for 4 days.

a major prebiotic used as a food ingredient.^{28,29} The substrate used in commercial endo-xylanase production should be the cheap sources of xylan such as corn cob, cane bagasse, and oil palm empty fruit bunch.^{30,31} However, those materials contain a great amount of cellulose, which may be converted into glucose in the presence of cellulases. Most of the xylanase producers exhibit the secretion of cellulases.^{11,32,33} The action of cellulases on cellulose produces glucose, which is readily consumed by the strain. Consequently, the accumulation of xylose in the medium represses the production of endo-xylanase. Therefore, the elimination of CCR in the strain used for commercial production of cellulases is necessary. The repression of hydrolase enzymes in fungi might be related to a regulatory gene, such as *creA* or *cre1*.³⁴⁻³⁶ In addition, deletion of *creA* in *A. oryzae* RIB40 increased the production of α -amylase in a liquid medium containing 1% glucose from less than 100 U/g dry weight in wild type to more than 1.100 U/g dry weight in mutant.³⁷ The same wild-type strain of *A. oryzae* grown in a medium containing 5% xylose produced almost negligible amounts of xylanase and β -glucosidase; however, the mutant strain grown in the same medium in which *creA* had been deleted produced approximately 40 and 0.6 U/g dry weight.³⁸ Therefore, *creA* can regulate the production of α -amylase, xylanase, and β -glucosidase.

Although the presence of glucose in the medium inhibited the production of endo-xylanase and β -xylosidase, it did not affect the growth of all strains. The strains grew well on both media, whether such strains contained glucose or not, and even the strains grew better in medium containing glucose. Considering that glucose can be easily metabolized by the cells, organisms might grow rapidly in a medium containing simple sugar than xylan. Therefore, the weight of biomass grown in the glucose medium was higher than that grown in the xylan medium. The higher the glucose concentration in the culture medium, the faster the growth of fungi because of the availability of nutrients to support growth (Fig. 3). This result was consistent with another report, that is, increasing glucose concentration in the medium could increase fungal biomass.³⁹

The pH of the medium after 4 days of incubation of all strains showed that the medium

added with glucose was lower compared with the xylan medium only. Considering that fungi also produce acid in the metabolism pathway, glucose is easily converted into acid compared with xylan. Therefore, the higher the glucose concentration in the medium, the more depleted the pH medium (data not shown). Other research also reported that increasing glucose at a concentration of 1%–3% in the medium would decrease the pH in *P. scabrosum*.³⁹

CONCLUSION

Among the strains examined, *A. aculeatus* FIG1 was the highest producer of endo-xylanase, and *T. asperellum* PK1J2 was the highest producer of β -xylosidase. The production of endo-xylanase and β -xylosidase was partially repressed in some strains and completely repressed in other strains when the strains were grown in a medium containing 1% glucose. However, the production of endo-xylanase and β -xylosidase was completely repressed when the strains were grown in a medium containing 3% glucose.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

RK performed the experiment, analyzed the data, and drafted the original manuscript. MNC, SJN, JW, DAS supervised and conceptualized the methodology, edited and reviewed the manuscript. All authors have read this manuscript and agreed to be published.

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DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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