BIODIVERSITAS
Volume 23, Number 12, December 2022
Pages: 6530-6538

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d231251

Xylanase production by *Trichoderma virens* MLT2J2 under solid-state fermentation using corn cob as a substrate

LUSTY ISTIQOMAH[1], MUHAMMAD NUR CAHYANTO[2,♥], ZUPRIZAL[3]

[1]Study Program of Biotechnology, Graduate School, Universitas Gadjah Mada. Jl. Teknika Utara, Sleman 55281, Yogyakarta, Indonesia
[2]Faculty of Agricultural Technology, Universitas Gadjah Mada. Jl. Flora No. 1, Bulaksumur, Sleman 55281, Yogyakarta, Indonesia.
♥email: mn_cahyanto@ugm.ac.id
[3]Department of Feed Nutrition, Faculty of Animal Science, Universitas Gadjah Mada. Jl. Fauna No. 3, Karanggayam, Sleman 55281, Yogyakarta, Indonesia

Manuscript received: 18 October 2022 2022. Revision accepted: 15 December 2022.

Abstract. *Istiqomah L, Cahyanto MN, Zuprizal. 2022. Xylanase production by* Trichoderma virens *MLT2J2 under solid-state fermentation using corn cob as a substrate. Biodiversitas 23: 6530-6538.* Xylanase is one of non-starch polysaccharide (NSP) degrading enzyme encompass in industrial applications like animal feed, food, biofuel, and textile. The objective of this study was to produce and characterize the crude xylanase from MLT2J2 isolate that was isolated from coconut husk. Molecular identification revealed that MLT2J2 isolate was identified as *Trichoderma virens*. The xylanase was produced from *Trichoderma virens* MLT2J2 (10^8 spores/g of corncob) using corn cob as substrate with 80% initial moisture content using under solid-state fermentation (SSF) at 30°C for 7 d of incubation. Changes in surface morphology and structure of fermented corn cob were monitored by Scanning Electron Microscope (SEM). Statistical analysis was performed using One-way analysis of variance (ANOVA) followed Tukey Kramer post hoc test to compare treatment means. Under fermentation parameters the maximum xylanase activity was 181.22 U/g-IDW, loss of dry matter 11.43%, and pH was decreased (4.11) at 5 d of incubation compared to initial pH (5.47). Xylanase was produced between a broad range pH 3-8 and showed acidophilic and mesophilic characteristics (optimum at pH 5,0 and 40°C), and also conserved more than 50% of activity before 4 h of incubation at 30°C and after 5 h at 40°C. The micrographs surface of corn cob before SSF appeared intact and smoother, while turned coarser after SSF, suggestive of a disrupted and more porous surface. These findings revealed that *T. virens* MLT2J2 could produce extracellular xylanase and potential to disrupt the cell wall of corn cob as a cheap substrate for enzyme production thereby increased the reactive surface area of corn cob for enzymatic in situ hydrolysis.

Keywords: Corn cob, enzyme characterization, solid-state fermentation, *Trichoderma virens*, xylanase

INTRODUCTION

Xylanase is one of the NSP (non-starch polysaccharide) degrading enzymes that hydrolyzes the β-1,4 glycosidic linkage of the backbone of the xylan polymeric chain which consists of xylose subunits and turns it into xylooligosaccharides (Sharma et al. 2019). The xylanases have become attractive due to their biotechnological applications in a wide range of industrial processes, covering all sectors of industrial enzymes markets i.e food, animal feed, waste treatment, ethanol production, textile, and pulp and paper industries (Basit et al. 2019). Several microbes produce xylanase (Verma and Satyanarayana 2012) and mesophilic fungi particularly genera *Trichoderma* and *Aspergillus* are considered as most potent xylanase producers than yeast, bacteria, and their equivalents from animal or vegetable sources and most widely used for commercial production (Steudler et al. 2012). The benefits include minor production costs, the option of large-scale production in industrial fermenters, an extensive variety of physical and chemical characteristics, the prospect of genetic manipulation, absence of effects brought about by seasonality, quick culture growth, and the use of non-burdensome methods (Poeta et al. 2018). These fungi produced xylanase extracellularly with a wide range of activities using various substrates. Despite *Trichoderma* sp. being among the prolific producers of xylanases, the high enzyme production cost and poor stability are major deterrents in widening their applications in the manufacturing industry. To date, xylanases producing *Trichoderma* species which include *Trichoderma harzianum* (Pathak et al. 2014; Lopez-Ramirez et al. 2018), *T. viride* (Soliman et al. 2021, Ja'afaru 2013; Ishida et al. 2020), *T. reesei* (Michelin et al. 2019; Hirasawa et al. 2018), *T. asperellum* UC1 (Ezeilo et al. 2019; Dorta-vásquez et al. 2019), *T. virens* (Tarayre et al. 2015; El-Shishtawy et al. 2015; Ngikoh et al. 2017), and *T. pleuroticola* (Korkmaz et al. 2017) have been studied. Inoculation of *T. virens* can degrade cellulose and hemicellulose in biomass (empty fruit bunches and palm oil mill effluent) hence speed up the composting process. *Trichoderma virens* has been known as a potential cellulase producer particularly endoglucanase, exoglucanase and β-glucosidase extracellularly for bioconversion processes and saccharification of agriculture wastes and also has good xylanase (β-xylosidase and endo-1,4-β-D-xylanase) activity (Tarayre et al. 2015; Ngikoh et al. 2017).

The fungal enzymes are greatly influenced by the nature, physical factors, and chemical composition of the carbon source (Peciulyte et al. 2014). The different kinds of

sources and chemical composition of biomass have an apparent influence on the secreted enzymes activities in cultures of fungal (Alvira et al. 2013) and previous studies reported several works on using corn cob (Soliman et al. 2012; Michelin et al. 2019), wheat bran and rice bran (Pathak et al. 2014; El-Shishtawy et al. 2015), sugarcane bagasse (Mahamud and Gomes 2012), oil palm empty fruit bunch and leaves (Ajijolakewu et al. 2016; Ngikoh et al. 2017; Ezeilo et al. 2019) as substrates for fermentation processes to produce hemicellulases. In this study, we used corn cob due to its high hemicellulose content and rich carbon, and also nitrogen sources for the growth and stimulation natural habitat of microorganisms (Elegbede and Lateef 2018). The application of corn cob as a solid substrate could generate value-added products and also lessen the undesirable impact on the environment.

To produce large-scale productions of fungal xylanase, substrate selection should be based on easy availability, conversion efficiency, toxic-free, and low operational cost (Ezeilo et al. 2019). In this study, the simplistic and cost-effective SSF method was selected to produce xylanase. The solid-state fermentation (SSF) process is a suitable and advantageous type of fermentation for enzyme production by filamentous fungi (Khanahmadi et al. 2018) and also more economical mainly due to the cheap and abundant availability of agricultural waste that can be used as substrates. Xylanases produced could be suitable for potential use in various industrial processes when scaled up by submerged or a lowly cost-effective solid-state fermentation for industrial and commercial purposes (Elegbede and Lateef 2018). Characterization of the crude enzyme clearly showed better activity and stability at optimum pH and temperature.

However, the characterization of the xylanase produced by *T. virens* was limited reported by previous studies. The objective of the study was to characterize the crude xylanase from *T. virens* MLT2J2 under solid-state fermentation using corn cob as a substrate and also as a xylanase inducer.

MATERIALS AND METHODS

Microorganism and inoculum preparation

The fungus was isolated from coconut husk and provided by Biotechnology Laboratory at the Department of Food and Agricultural Product, Universitas Gadjah Mada. The fungus strain MLT2J2 was chosen from a group of fungi with endo-xylanase activity (0.16 U/ml) (Krisnawati et al. 2022). The fungus culture was propagated on potato dextrose agar (PDA) medium at 30°C, kept on a PDA agar slant at 4°C, and lyophilized at -80°C for long time storage. The inoculum was prepared by maintaining the fungus on PDA plates at 30°C for 7 d. Spores suspensions were prepared by adding 10 mL 1% Tween 80 (v/v) to slant cultures and the surface was gently scraped with a sterilized pipette. The spores were counted in a Neubauer counting chamber under a light microscope with 200 x magnification and counted by hand counter.

Identification of microorganism

Strain MLT2J2 was identified based on the morphological characteristics of conidiophores and conidia, using the taxonomic keys (Srinivasa et al. 2014). Sample of fully sporulated strains culture was mounted on slides and examined under a compound microscope (Olympus) to observe morphological structures.

Molecular identification was performed based on partial genetic analysis in the locus of Internal Transcribed Space (ITS) ribosomal DNA. The fungus was grown in potato dextrose broth (PDB) medium and incubated for 72 h. Mycelium was harvested for the DNA extraction process using nucleon PHYTOpure reagent (Amersham LIFE SCIENCE). Amplification PCR on ITS using primer ITS 4: 5' - TCC TCC GCT TAT TGA TAT GC - 3' and primer ITS 5: 5' - GGA AGT AAA AGT CGT AAC AAG G - 3' (Khan and Bhadauria 2019). Purification of PCR product was determined by the PEG precipitation method (He et al. 2013) followed by sequencing cycles. Analysis of nitrogen base sequence using automated DNA sequencer (ABI PRISM 3130 genetic analyzer) (Applied Biosystem) conducted by 1st BASE (Genetika Science).

Raw data were trimmed and assembled using the BioEdit program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Sequence data then BLAST with registered genome data in NCBI/National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/) to determine the species with the highest and closest similarity/homology molecularly. A phylogenetic tree was constructed using the Maximum-likelihood model of the MEGA 7 program (Kumar et al. 2016). The stability of grouping was measured using 2000 bootstrap replicates.

Chemical composition

The chemical composition of corn cob (CC) consists of dry matter, crude protein, crude fiber, and lipid were determined according to AOAC (2005). The lignocellulose content (cellulose, hemicellulose, lignin) CC was analyzed according to Van Soest et al. (1991).

Production of xylanase under SSF

Corn cob powder was used as a substrate for enzyme production. SSF was conducted according to modified method of Ezeilo et al. (2019) in 250 mL Erlenmeyer flask at 30°C, containing 6 g of CC moistened with 24 mL of a modified Mandels medium with 0.3 g/L urea, 1.4 g/L $(NH_4)_2SO_4$, 2 g/L KH_2PO_4, 0.3 g/L $CaCl_2$, 0.3 g/L $MgSO_4$, 0.005 g/L $FeSO_4$, 0.0016 g/L $MnSO_4 \cdot H_2O$, 0.0014 g/L $ZnSO_4 \cdot 7H_2O$, 0.002 g/L $CoCl_2$, 0.75 g/L peptones and 2 mL/L Tween 80 (pH 5.2) to obtain a final moisture level of 80% (Mandels and Weber 1969). Moisture content was determined according to AOAC (2005). The flasks were autoclaved (15 min at 121 C, 20 psi) and left to cool at room temperature. Fermentation was initiated by inoculating the flask with 1 mL inoculum (108 spores/g of corncob) and incubated for 7 d at 30°C. The experiments were triplicate. The fermented substrate samples were taken every 24 h for xylanase activity.

Crude xylanase extraction

In 24 h intervals, 1 g of CC substrate respectively was taken out from the flask in order to measure the pH value using pH meter (Thermo Scientific Orion Star A111) and dried in order to determine the moisture content (AOAC 2005). Enzyme extraction was performed according to Khanahmadi et al. (2018). Four grams of fermented substrate of each flask was extracted by adding 50 mL of 0.05 M citrate buffer (pH 5.3) to residual content and then mixed on a rotary shaker at 200 rpm for 2 h. The mixture was then centrifuged at 5000 rpm in a refrigerated centrifuge (Eppendorf 5810R, Germany) for 15 min. The supernatant was then filtered through a 0.45 µm membrane (Axiva). The clear supernatant was temporarily stored at 4°C and used for measuring xylanase assay.

Xylanase assay

Beechwood xylan was prepared by weighed 1.0 g of xylan into a 120 mL beaker glass and 4 mL of 95% ethanol was added to wet the sample. A magnetic stirrer bar was added and followed by 90 mL of distilled water while stirred the slurry on a hot-plate magnetic stirrer. The heat setting was adjusted to 120°C and stirred vigorously then covered the beaker loosely with aluminum foil and continue stirred vigorously and turn the heat off when the solution began to boil and kept stirring the solution until the xylan completely dissolves (approx. 10 min). Adjust the volume of the solution to 100 mL.

Xylanase activity was determined by measuring the rate of reducing sugar released from 1% (w/v) beechwood xylan in 0.1 M acetate buffer (pH 5.0) (Khanahmadi et al. 2018). The mixture contained 0.1 mL crude enzyme and 1 mL of 1% (w/v) xylan solution. The tubes were covered and incubated at 40°C for 20 min in a water bath. Then 0.5 mL of dinitrosalcylic acid (DNS) reagent was added to each tube to stop the reaction and placed in boiling water bath exactly for 10 min. After cooling the samples in a cold water (approx. 10 min), the absorbance was read at 540 nm in spectrophotometer (Agilent Cary 60 UV-Vis) and amount of the reducing sugar liberated was measured by DNS method (Miller 1959) using xylose solution (0 - 0.4 mg/mL) as standard reference. One unit of xylanase activity (U) was defined as the amount of enzyme that released 1 µmol xylose per min under the assay conditions. Xylanase activity was expressed as U per gram of fermented dry matter (U/-IDW).

Assay for physic-chemical properties of enzymes

Optimum pH and temperature

The optimum pH of the fungal xylanase under SSF conditions was examined by varying the pH of reaction mixtures from 3 to 8. The buffers (0.1 M) used were acetate buffer (pH 3-5) and phosphate buffer (pH 6-8). The activity of the crude xylanase was assessed for temperatures between 30-70°C with 10°C intervals at the previous optimum pH. Results were expressed as the % of relative activity (Ezeilo et al. 2019).

Xylanase stability

Temperature stability was performed by incubating the crude enzyme at 30 and 40°C for 0-5 h, at 30 min intervals. The activity of the crude xylanase was assessed at the previous optimum pH (pH 5.0). Results were expressed as the % of relative activity (Ezeilo et al. 2019).

Scanning electron microscopy (SEM) on UCC and FCC

Two grams of UCC and FCC were suspended in an Erlenmeyer flask containing 50 mL of distilled water, agitated at 150 rpm for 30 min, and filtered through a Whatman No 1 filter paper then the residues were dried in an oven at 70°C for 6 h (Ezeilo et al. 2019). The sample was swab onto each carbon tape and dried, then sputter-coated with a thin gold film (Hitachi MC1000) to avoid charging under the electron beam, and then analyzed under SEM (Hitachi SU3500 02-02) operating at 5 kV and electric current of 108 µA under 2000 x magnification.

Data analysis

Data of xylanase activity, pH value, loss of dry matter, and moisture content were analyzed statistically by One-way ANOVA, in which the level of significance of 0.05 ($\alpha<0.05$), followed by the Tukey Kramer post hoc test to compare treatment means using Costat software (Cohort 2008). The triplicate data are presented as mean ± standard deviation.

RESULTS AND DISCUSSION

Identification of microorganism

The strain of the genus *Trichoderma* isolated from coconut husk showed predominantly effuse conidiation without formation of any pustule. This isolate produced yellow pigment on PDA. Conidial color change was observed from white to varying shades of green and these conidia were formed by 48 h and turned green within 72 h (Figure 1.A). These characteristics coincide with those described by Sharma and Singh (2014). Kumar et al. (2020) reported that conidial wall of *T. virens* was smooth with grayish-green conidial color and the presence of chlamydo-spores with spore germination time around 12 hours. Mycelial form was floccose to arachnoid with watery white color.

Morphological study revealed two types of arrangement of conidiophores and phialides. Regarding the microscopic characteristics (Figure 1.B) of the *Trichoderma* colonies, the strain identified in the present study had conidiophores with the following general characteristics: smoothly bent, gather and not spread to top. Conidial shape was obovoid to broadly ellipsoidal, both ends broadly rounded or with the base narrower. Length and width of conidia was 2.93 to 3.96 µm. Phialide shape was ampulliform and hung like banana in the conidiophore, base and apex were narrower than the middle. These characteristics were in agreement with the findings of earlier researchers (Sharma and Singh 2014; Srinivasa et al. 2014).

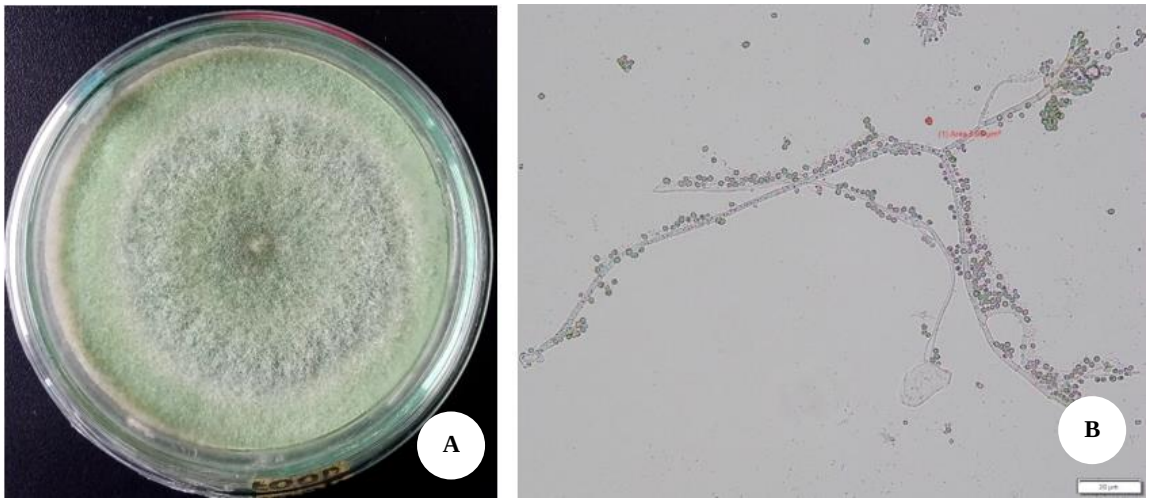

Figure 1. Macroscopic characteristics of *Trichoderma* MLT2J2 at 5 d with white mycelium on PDA media (A) and microscopic characteristic with conidiophores, conidia of ovoid shape, and phialides (B)

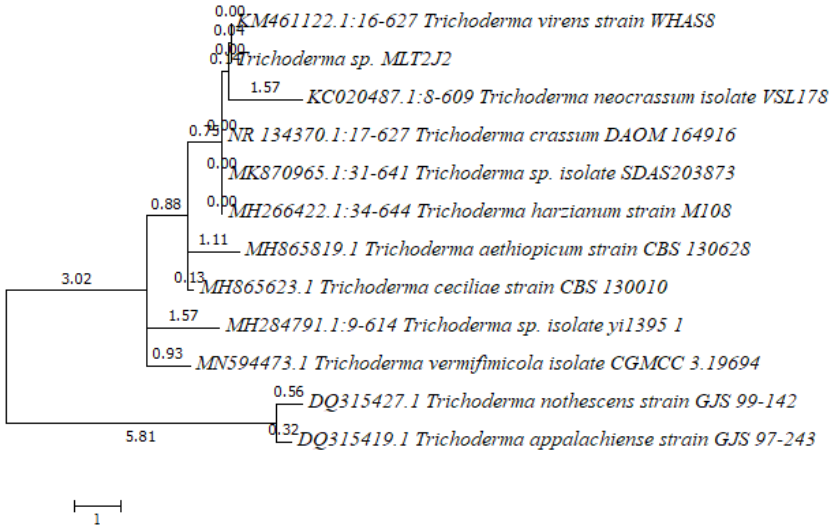

Figure 2. Phylogenetic tree of MLT2J2 isolated from coconut husk based on partially genetic analysis in locus of Internal Transcribed Space (ITS) ribosomal DNA

Molecular identification of fungus strain by BLAST analysis demonstrated that MLT2J2 isolate was identified as *Trichoderma virens* in DNA bank database with 100% of maximum identity, 1131 of maximum score, 1131 of total score, 100% of query coverage, and 0.0 of E-value. Phylogenetic analysis of MLT2J2 isolate by nucleotide references from Gene Bank data in NCBI was shown in Figure 2. The tree describes the relationship between selected sequences retrieved from the GenBank database and nucleotide sequences in this study. The phylogenetic tree showed that *T. virens* MLT2J2 isolated from coconut husk was closely related to *Trichoderma virens* strain WHAS8 (accession number: KM461122.1) based on partially genetic analysis in locus of Internal Transcribed Space (ITS) ribosomal DNA.

This fungus was classified in the Kingdom of Fungi, Phylum of Ascomycota, Subphylum of Sordariomycetes, Order of Hypocreales, Family of Hypocreaceae, Genus of Trichoderma, and Species of *T. virens*. El-Shishtawy et al. (2015) reported that xylanase production by *T. virens* initially was low and at 72 h a sharp increase in enzyme production was observed and after that, the enzyme activity remained constant. Xylanase activity of *T. virens* UKM1 during 7 days of incubation in the medium was 230.62 U/mL (Ngikoh et al. 2017). A study from Tarayre et al. (2015) revealed that mold isolated from termite *Reticulitermes santonensis* produced xylanase (426 IU/mL) and was identified morphologically as *T. virens* strain CTGxAviL.

Chemical composition of corn cob

Corn cob is a by-product of the corn industry used as animal feed, which is an appropriate substrate for the SSF process including xylanase production due to its physical characteristic and hemicellulose content. Hemicelluloses are hydrophilic, especially xylan, and make them responsible for their non-affinity towards oils and fats (Louis and Venkatachalam 2020). The chemical composition of the corn cob powder shown in Table 1.

Olagunju et al. (2013) reported nutrient content of corn cob contained 5.42% moisture, 4.46% ash, 9.96% fat, 4.10% crude protein, 7.72% fiber, and 74.51 carbohydrates. Castillo et al. (2021) stated that corn cob contained 1.32% moisture, 2.58% ash, 1.22% fat, 2.42% protein, 12.16% crude fiber, and 81.63% total carbohydrates. Louis and Venkatachalam (2020) reported the composition of corn cob powder as follows: 45.01% cellulose, 33.12 % hemicellulose, 13.81% lignin, 3.1% ash, and 4.96% other extractives. Corn cob has the highest *xylan content* than other lignocellulose waste and consists about 35% of *xylan* (Gowdhaman and Ponnusami 2015). The chemical content of the biomass are varies depending on their geographical location such as sunny hours, temperature, seasonal changes, environmental, and climatic conditions (Delfine et al. 2017; Liu et al. 2018; AL-Hmadi et al. 2021), still it was found to be in range reported in the literature (Jekayinfa et al. 2020).

Effect of incubation time

Xylanase activity, pH value, loss of dry matter, and moisture content during 7 days of incubation under SSF of CC substrate by *T. virens* MLT2J2 were observed at 30°C. Referring to Figure 3.A, the maximum xylanase activity resulted from 5-d fermentation of CC was 181.22 U/g-IDW. Xylanase production at 6 d of incubation decreased sharply (107.49 U/g-IDW). The decrease of enzyme yields may be due to the clumping of solid particles which results in the decrease in inter-particle space leading to decreased diffusion of nutrients (El-Shishtawy et al. 2015).

Trichoderma virens strain CTGxAviL produced the highest β-xylosidase activity (0.38 IU/ml and endo-1,4-β-D-xylanase activity (426 IU/ml) after 72 h and 96 h of fermentation on *αC α*-cellulose, respectively (Tarayre et al. 2015). El-Shishtawy et al. (2015) also observed that initially xylanase production from *T. virens* using alkali pre-treated wheat bran as a substrate of SSF was low and sharp increased at 72 h of incubation at 30°C with maximum production 348 U/g solid. Xylanase of *T. virens* performed maximum activity at 4 d of incubation (75 U/g solid) using sugarcane bagasse as a solid substrate (Mohamed et al. 2013). The highest xylanase activity was found after 144 h of incubation which was 13.25 U/ml (Mahamud et al. 2012). Ezeilo et al. (2019) reported that xylanase production was maximal at the 6th day of incubation. Longer SSF incubation times were previously reported to give maximum quantities of xylanases. Xylanase production increased steadily with an increase in fermentation time (El-Shishtawy et al. 2015).

Figure 3.B showed that fermentation time affected the pH value of the solid medium ($p<0.05$). Initial pH was 5.47 then decreased at 2nd d (4.18) and there was no difference in pH until 7 d of incubation ($p>0.05$). The changes in pH in the fermentation medium during the SSF process were due to microbial metabolism. In the case of *A. niger* CCUG33991, the pH of the fermentation medium was dropped after 24 h of fermentation and no pH increase occurred in a corn cob and sorghum stover, which is low in protein content (Khanahmadi et al. 2018). The most reasonable explanation for this pH reduction could be the secretion of organic acids such as citric, acetic, or lactic acids in the fermentation medium (Vandenberghe et al. 1999). Based on the reduction of readily available carbon sources, consumption of produced acids and carbon skeleton of proteins may start and the latter releases ammonium salts. Both acid consumption and ammonium release will increase pH value (Dahlquist 2013).

Dry matter loss is an indication of metabolic extent in SSF. Figure 3.C showed that there were high losses in dry matter of CC at 5 days of SSF. Losses of dry matter are a combination of reduced nutrient concentration and carbon dioxide production and other volatiles during fermentation (Ferraretto et al. 2018). This result is similar to Khanahmadi et al. (2018) who stated that high losses in dry matter of CC indicated extensive metabolism and growth of the fungus on this substrate and may justify high xylanase production. After 5 days of incubation, dry matter loss decreased due to fungal activity slows and fermentation stabilizes caused less substrate (hemicellulose) degradation and less-metabolizing water-soluble sugars (Goeser et al. 2015). During the fermentation, *T. virens* produced reducing sugars as the depolymerized products of hemicellulose catalysed by xylanase and conversion of carbohydrates and sugars to organic acids (acetic acid, propionic acid, and lactic acid) (Ferraretto et al. 2018). The contents of acetic and butyric acids are primary negative indicators of the quality of the fermentation process that showed marked losses of dry matter and energy during fermentation (Agarussi et al. 2019).

Table 1. Nutrient content of corn cob (100% of dry matter)

Components	Nutrient content (%)
Moisture	7.40 ± 0.11
Ash	1.74 ± 0.02
Lipid	1.10 ± 0.00
Crude protein	1.86 ± 0.00
Crude fiber	0.39 ± 0.03
Cellulose	45.68 ± 0.01
Hemicellulose	41.03 ± 0.06
Lignin	6.38 ± 0.23

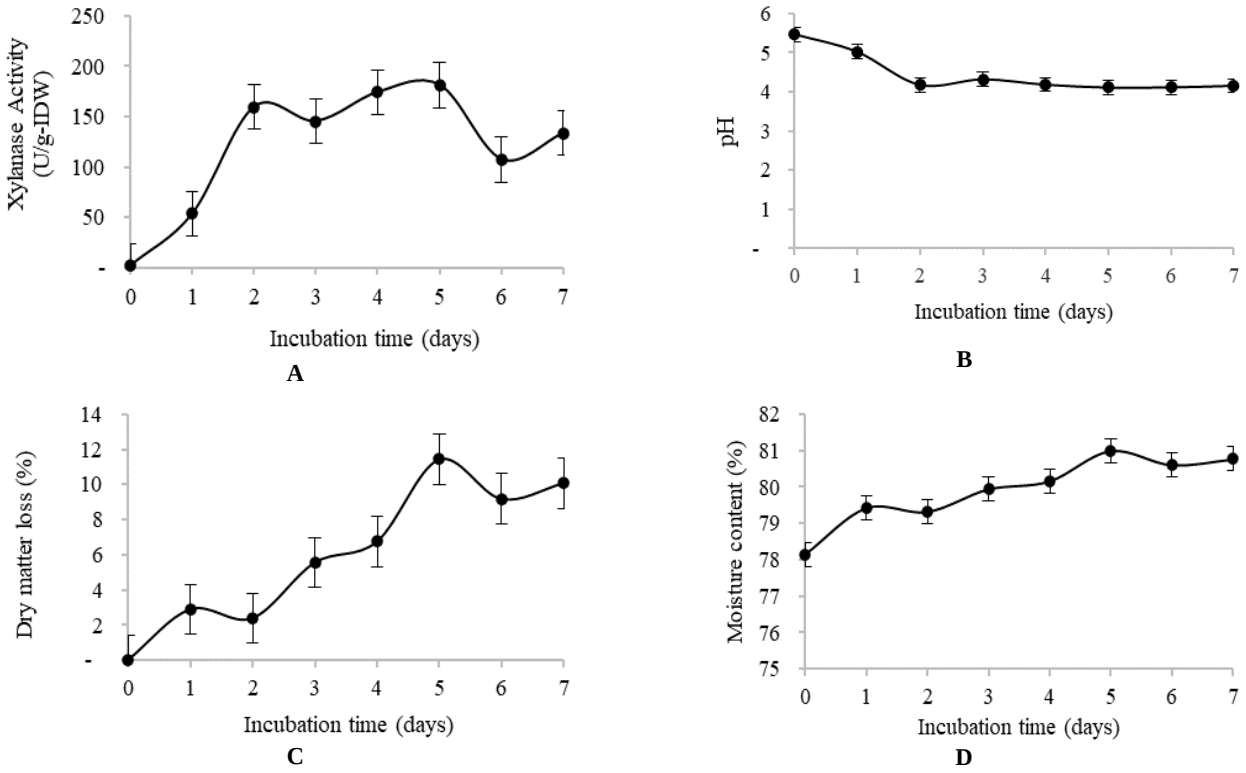

Figure 3. Effect of time fermentation of corn cob on xylanase production (a), pH (b), loss of dry matter (c), and moisture content (d) under SSF flask culture by *T. virens* MLT2J2 at incubation temperature 30 °C and initial moisture content of 80%. Mean ± Stdev of three replications

The moisture content is one of the most important factors that influence the growth of microorganisms and thereby product yield (enzyme production) in SSF. Moisture content increased during fermentation ($p<0.05$). Initial moisture content was 78.15% then increased up to 80.99% in line with xylanase production at 5 days of incubation (Figure 3.D), after which a reduction of moisture was observed. More extensive metabolism in CC caused higher dry matter loss and metabolic water release of which increased moisture content. Moisture can cause swelling of the substrates, therefore facilitating better utilization of the substrate by microorganisms (El-Shishtawy et al. 2015). Water evaporation, microbial consumption, often cause obvious available water loss, thus, it is necessary to replenish the lost water caused by moisture evaporation and microbial utilization in SSF (He et al. 2019). After 5 days in SSF, the moisture content decreased causing reduction in solubility of the nutrients of the substrate, low degree of swelling, and high water tension (Mohamed et al. 2013). On the contrary, moisture content dropped in cultures provided with corn cob as substrate due to substrate evaporation (Khanahmadi et al. 2018).

Parameters affecting activity and stability of crude xylanase

Xylanase enzyme at 7-day of incubation produced by *T. virens* MLT2J2 under an optimized SSF condition was characterized, and the results are discussed in the following subsections.

Effect of pH

Effect of pH on the activity of the enzymes was investigated between pH 3.0-8.0. Results revealed that enzyme activities were maximum within a narrow range of pH 4.0-5.0 (Figure 3). This study found that pH 5.0 was significant in maximizing activities of the xylanase components. *Trichoderma virens* MLT2J2 showed reduced relative activity of xylanase below 50% for pH 7.0-8.0. This result is in agreement with previous studies that the most fungal xylanase activities are optimum at moderately acidic to alkaline pH range; for instance, pH 3-4 for *T. asperellum* UC1 (Ezeilo et al. 2019), pH 5,0 for *T. harzianum* (Ahmed et al. 2012), pH 5.3 for *T. asperellum* USM SD4 xylanase (Ajijolakewu et al. 2016), pH 6.0 for *T. reesei* MUM 97.53 (Michelin et al. 2019), and pH 9-10 for *Sarocladium kiliense* (Tarayre et al. 2015).

Effect of temperature

Enzymes are typically sensitive to extreme temperature conditions thus, identifying the best process settings for the highest enzyme activity will boost performance of the enzymatic processes. The xylanase produced shows satisfying properties as regards temperature. Figure 4 depicts the effect of temperature on xylanase activities, investigated at temperatures 30-70°C. This study revealed that optimum xylanase activity was reached at 40°C.

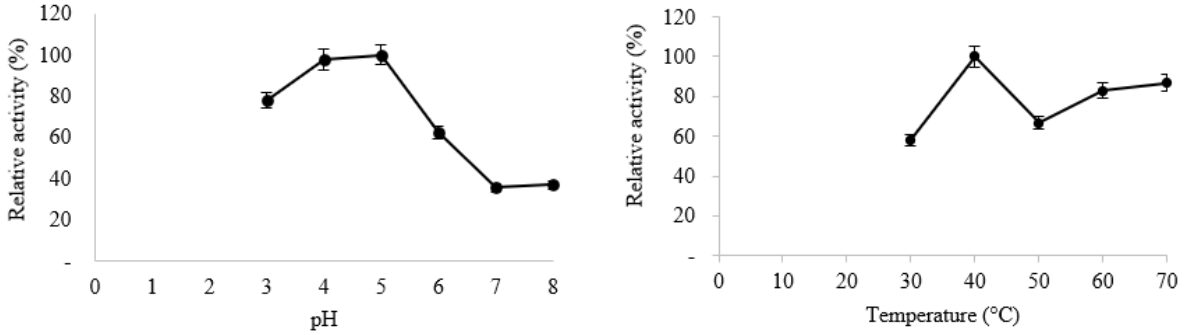

Figure 4. Optimum pH (left) and temperature (right) of xylanase expressed in terms of relative activity (%). The highest enzyme activity was taken as 100%. Experiments on optimum temperature were investigated at constant pH (5.0), while experiments on optimum pH were investigated at constant temperature (40 °C).

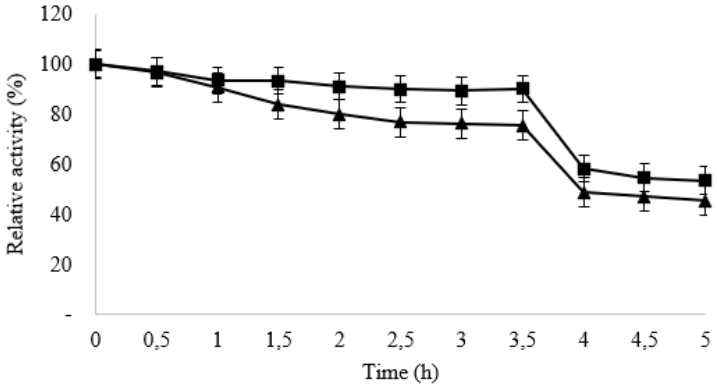

Figure 5. Temperature stability of xylanase produced by *T. virens* MLT2J2 assessed at (▲) 30 °C and (■) 40 °C

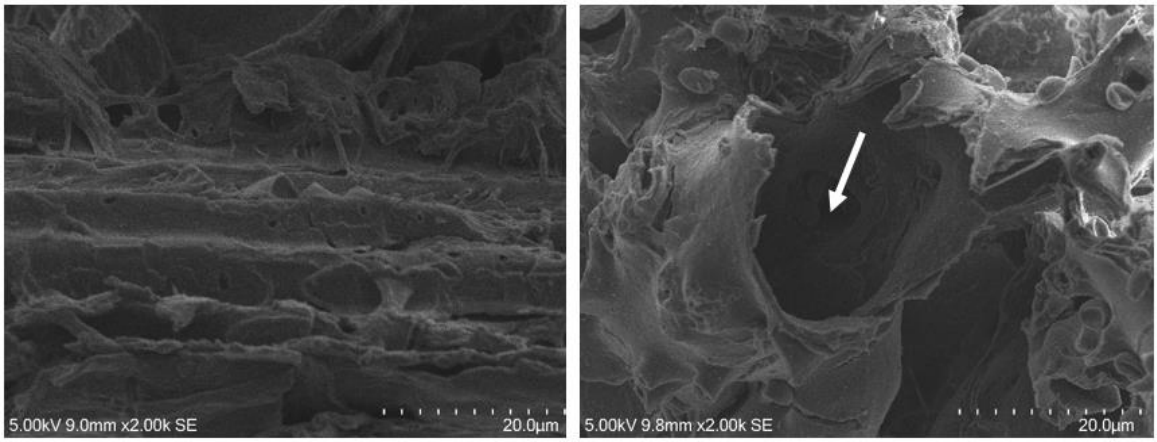

Figure 6. SEM micrographs of corn cob before SSF (left); after SSF (right) under 2000 × magnification. White arrows indicated the presence of pores

Apparently, activity of xylanase declined with increasing incubation temperature. But the xylanase was less heat labile and retained 59% of its relative activity at 50°C. The declining residual enzyme activity over time was indicative of progressive denaturation of the enzyme active sites in response to the change in environmental temperature. This also showcased the ability of xylanase of *T. virens* MLT2J2 to function well under thermophilic conditions (60-70°C) conserved 79% and 84% of activity, respectively and thus suggestive of their possible industrial and commercial applications.

The optimum temperature for xylanase activity from different fungal species is similar to or slightly higher than the results obtained; for instance, 50°C for *T. viride* (Ja'afaru 2013) and *T. harzianum* (Ahmed et al. 2012), and 60°C for *T. reesei* MUM 97.53 (Michelin et al. 2019), *T. harzianum* PPDDN-10 NFCCI-2925 (Pathak et al. 2014), and *S. kiliense* (Tarayre et al. 2015).

Xylanase stability

Despite the high temperature optimum of enzymes, the thermostability of an enzyme is also a very important property because at optimum temperature, it should be stable, i.e., it should not get denatured before the desired reaction time (Pathak et al. 2014) and the temperature stability has a marked influence on enzymatic applications and storage (Ezeilo et al. 2019). Figure 5 demonstrated that xylanase produced by *T. virens* conserved more than 50% of activity after 5 h at optimum temperature (40°C). However, it conserved less than 50% of activity after 4 h at 30°C.

This stability was higher than the previously reported value of about 50% loss for 6 h at 60°C and 20-24% activities were lost when kept at 55°C for 5 h (Pathak et al. 2014). Tarayre et al. (2015) reported that xylanase produced by *S. kiliense* conserved more than 90% of activity after 30 min at 80°C and the enzymatic activity decreased to 40% at 80°C. Ajijolakewu et al. (2016) revealed that the half-lives of xylanase of *T. asperellum* USM SD4 was 1 h at 50°C. The enzyme gave good temperature stability up to 6 h at 60°C (approximately 50 % retained), and 20-24% activities were lost when kept at 55°C for 5 h. Ezeilo et al. (2019) reported that *T. asperellum* UC1 at 30°C and 40°C showed remarkable stability of xylanase with their half-lives reaching 19.26 h at 40°C, 16.16 h at 50°C and 15.16 h at 60°C. This observation makes our strain quite original. However, such a temperature causes denaturation if the heating time is too long.

Surface morphology of CC

Changes in surface morphology and structure of fermented CC were monitored by SEM. Figure 6 displayed the micrographs of CC before and after SSF. SEM results showed that enzymatic treatment greatly influenced the microstructure of the WB samples, irrespective of their particle size. The surface of CC appeared intact and smoother before SSF but turned coarser after SSF, suggestive of a disrupted and more porous surface. Similar morphological changes in other SSF substrates have been described (Ezeilo et al. 2019). Xue et al. (2020) reported that treated wheat bran using xylanase exhibited a more porous structure than untreated ones.

In conclusion, crude enzyme by a *T. virens* MLT2J2 was found to comprise xylanases and demonstrated desirable industrial properties including good thermal stabilities and displaying a wide pH range (3-8). The findings advocated the suitability of raw corn cob (CC) as a cheap substrate for enzyme production by *T. virens* MLT2J2. The produced enzyme was found efficient in hydrolyzing the CC, indicating their potential in sustainable utilization for the remediation of xylan-containing agro-industrial biomass into value-added products.

ACKNOWLEDGEMENTS

The authors gratefully thank Indonesian Ministry of Research and Technology for SAINTEK scholarship funding awarded to LI.

REFERENCES

Agarussi MCN, Pereira OG, de paula RA, da silva VP, Roseira JPS, e silva FF. 2019. Novel lactic acid bacteria strains as inoculants on alfalfa silage fermentation. Sci Rep 9: 8007. DOI: 10.1038/s41598-019-44520-9.

Ahmed S, Imdad SS, Jamil A. 2012. Comparative study for the kinetics of extracellular xylanases from *Trichoderma harzianum* and *Chaetomium thermophilum*. Electron. J Biotechnol 15: 1-8. DOI: 10.2225/vol15-issue3-fulltext-2.

Ajijolakewu KA, Leh CP, Wan Abdullah WN, Lee CK. 2016. Assessment of the effect of easily-metabolised carbon supplements on xylanase production by newly isolated *Trichoderma asperellum* USM SD4 cultivated on oil palm empty fruit bunches. BioResources 11: 9611-9627. DOI: 10.15376/biores.11.4.9611-9627.

AL-Hmadi H, El Mokni R, Joshi RK, Ashour ML, Hammami S. 2021. The impact of geographical location on the chemical compositions of *Pimpinella lutea* desf. growing in Tunisia. Appl Sci 11: 7739. DOI: 10.3390/app11167739.

Alvira P, Gyalai-Korpos M, Barta Z, Oliva JM, R´eczey K, Ballesteros M. 2013. Production and hydrolytic efficiency of enzymes from *Trichoderma* reesei RUTC30 using steam pretreated wheat straw as carbon source. J Chem Technol Biotechnol 88: 1150-1156. DOI: 10.1002/jctb.3955.

AOAC (Association of Official Agricultural Chemists). 2005. Official Methods of Analysis. Assoc Off Anal Chem Arlington.

Basit A, Jiang W, Rahim K. 2019. Xylanase and its industrial applications. In: Thalita PB, Basso TO, Basso LC (eds). Biotechnological Applications of Biomass. IntechOpen, London. DOI: 10.5772/intechopen.89320.

Castillo S, Rodríguez A, Bautista-Villarreal M, García-Solano N, Gallardo-Rivera C, Báez-González JG, Sánchez-García E, García-Alanis KG. 2021. Rheological and nutritional characterization of sweet corn by-product (cob) to develop a functional ingredient applied in dressings. Front Nutr 8: 666654. DOI: 10.3389/fnut.2021.666654.

Cohort. 2008. CoSTAT Version 6.400. Cohort Software 798. Lighthouse Ave, Monterey, USA.

Dahlquist E. 2013. Technologies for Converting Biomass to Useful Energy Combustion, Gasification, Pyrolysis, Torrefaction and Fermentation. CRC Press, Taylor & Francis Group, London. DOI: 10.1201/b14561.

Delfine S, Marrelli M, Conforti, F, Formisano C, Rigano D, Menichini F, Senatore F. 2017. Variation of *Malva sylvestris* essential oil yield, chemical composition and biological activity in response to different environments across Southern Italy. Ind Crop Prod 98: 29-37. DOI: 10.1016/j.indcrop.2017.01.016.

Dorta-vásquez R, Valbuena O, Pavone-maniscalco D. 2019. Solid-state fermentation of paper sludge to obtain spores of the fungus *Trichoderma asperellum*. The EuroBiotech J 3: 71-77. DOI: 10.2478/ebtj-2019-0008.

Elegbede JA, Lateef A. 2018. Valorization of corn-cob by fungal isolates for production of xylanase in submerged and solid state fermentation media and potential biotechnological applications. Waste Biomass 9: 1273-1287. DOI 10.1007/s12649-017-9932-y.

El-Shishtawy RM, Mohamed SA, Asiri AM, Gomaa A-b M, Ibrahim HI, Al-Talhi HA. 2015. Saccharification and hydrolytic enzyme production of alkali pre-treated wheat bran by *Trichoderma virens* under solid state fermentation. BMC Biotechnol 15: 37. DOI 10.1186/s12896-015-0158-4.

Ezeilo UR, Tin C, Huyop F, Izzati I, Abdul R. 2019. Raw oil palm frond leaves as cost-effective substrate for cellulase and xylanase productions by *Trichoderma asperellum* UC1 under solid-state fermentation. J Environ Manag 243: 206-217. DOI: 10.1016/j.jenvman.2019.04.113.

Ferraretto LF, Fernandes T, Filho WIS, Sultana H, Moriel P. 2018. Dry matter loss, fermentation profile, and aerobic stability of wet brewers grains ensiled with various amounts of dry ground corn. Professional Anim Sci 34: 642-648. DOI: 10.15232/pas.2018-01761.

Goeser JP, Heuer CR, Crump PM. 2015. Forage fermentation product measures are related to dry matter loss through meta-analysis. Professional Anim Sci 31: 137-145. DOI: 10.15232/pas.2014-01356.

Gowdhaman D, Ponnusami V. 2015. Production and optimization of xylooligosaccharides from corncob by *Bacillus aerophilus* KGJ2

xylanase and its antioxidant potential. Intl J Biol Macromol 79: 595-600. DOI: 10.1016/j.jenvman.2019.04.113.

He Q, Peng H, Sheng M, Hu S, Qiu J, Gu J. 2019. Humidity control strategies for solid-state fermentation: capillary water supply by water-retention materials and negative-pressure auto-controlled irrigation. Front Bioeng Biotechnol 7: 263. DOI: 10.3389/fbioe.2019.00263.

Hirasawa H, Shioya K, Furukawa T, Sumitani J-I, Kawaguchi T, Morikawa Y, Shida Y, Ogaswara W. 2018. Engineering of the *Trichoderma reesei* xylanase3 promoter for efficient enzyme expression. Appl Microbiol Biotechnol 102: 2737-2752. DOI: 10.1007/s00253-018-8763-5.

Ishida T, Parks JM, Smith JC. 2020. Insight into the catalytic mechanism of GH11 xylanase: computational analysis of substrate distortion based on a neutron structure. J Am Chem Soc 142: 17966-17980. DOI: 10.1021/jacs.0c02148.

Ja'afaru MI. 2013. Screening of fungi isolated from environmental samples for xylanase and cellulase production. Intl Scholarly Res Notices 2013. DOI: 10.1155/2013/283423.

Jekayinfa SO, Orisaleye JI, Pecenka R. 2020. An assessment of potential resources for biomass energy in Nigeria. Resources 9: 1-41. DOI: 10.3394/resources9080092.

Khan AM, Bhadauria S. 2019. Molecular characterization of keratin degrading fungi isolated from semi-arid soil by PCR using ITS4 and ITS5 primers. J King Saud Univ - Sci 31: 1418-1423. DOI: 10.1016/j.jksus.2018.04.014.

Khanahmadi M, Arezi I, Amiri M, Miranzadeh M. 2018. Bioprocessing of agro-industrial residues for optimization of xylanase production by solid- state fermentation in flask and tray bioreactor. Biocatal Agric Biotechnol 13: 272-283. DOI: 10.1016/j.bcab.2018.01.005.

Korkmaz MN, Ozdemir SC, Uzel A. 2017. Xylanase production from marine derived *Trichoderma pleuroticola* 08ÇK001 strain isolated from Mediterranean coastal sediments. J Basic Microbiol 2017: 1-13. DOI: 10.1002/jobm.201700135.

Krisnawati R, Sardjono, Widada J, Suroto DA, Cahyanto MN. 2022. Effect of glucose on endo-xylanase and β-xylosidase production by fungi isolated in Indonesia. J Pure Appl Microbiol (1): 226-234. DOI: 10.22207/JPAM.16.1.12.

Kumar S, Stecher G, Tamura K. 2016. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets brief communication. Mol Biol Evol 33: 1870-1874. DOI:10.1093/molbev/msw054.

Kumar G, Singh A, Pandey S, Singh J, Chauhan SS, Srivastava M. 2020. Morphomolecular identification of *Trichoderma* sp. and their mycoparasitic activity against soil borne pathogens. IJBSM 11: 613-627. DOI: 10.23910/1.2020.2131.

Liu Y, Chen P, Zhou M, Wang T, Fang S, Shang X, Fu X. 2018. Geographic variation in the chemical composition and antioxidant properties of phenolic compounds from *Cyclocarya paliurus* (Batal) iljinskaja leaves. Molecules 23: 2440. DOI: 10.3390/molecules23102440.

Lopez-Ramirez N, Volke-Sepulveda T, Gaime-Perraud I, Saucedo-Castañeda G, Favela-Torres E. 2018. Effect of stirring on growth and cellulolytic enzymes production by *Trichoderma harzianum* in a novel bench-scale solid-state fermentation bioreactor. Bioresour Technol 265: 291-298. DOI: 10.1016/j.biortech.2018.06.015.

Louis ACF, Venkatachalam S. 2020. Energy efficient process for valorization of corn cob as a source for nanocrystalline cellulose and hemicellulose production. Intl J Biol Macromol 163: 260-269. DOI: 10.1016/j.ijbiomac.2020.06.276.

Mahamud M, Gomes D. 2012. Enzymatic saccharification of sugar cane bagasse by the crude enzyme from indigenous fungi. J Sci Res 4: 227-238. DOI:10.3329/jsr.v4i1.7745.

Mandels M, Weber J. 1969. The production of cellulases. Adv Chem Ser 95: 391-414. DOI: 10.3329/jsr.v4i1.7745.

Michelin M, Mota AMO, Silva DP, Ruzene DS, Vicente AA, Teixeira JA. 2019. Production of biomass-degrading enzymes by *Trichoderma reesei* using liquid hot water-pretreated corncob in different conditions of oxygen transfer. BioEnergy Res 12: 583-592. DOI: 10.1007/s12155-019-09991-8.

Miller G. 1959. Use of dinitrosaiicyiic acid reagent for determination of reducing sugar. Anal Chem 31: 426-428. DOI: 10.1021/ac60147a030.

Mohamed SA, Al-malki AL, Khan JA, Kabli SA, Al-garni SM. 2013. Solid state production of polygalacturonase and xylanase by *Trichoderma* species using cantaloupe and watermelon rind. J Microbiol 51: 605-611. DOI: 10.1007/s12275-013-3016-x.

Ngikoh B, Karim NAA, Jahim J, Bakar FDA, Murad AMA. 2017. Characterisation of cellulases and xylanase from *Trichoderma virens* UKM1 and its potential in oil palm empty fruit bunch (OPEFB) saccharification. J Phys Sci 28: 171-184. DOI: 10.21315/jps2017.28.s1.11.

Olagunju A, Onyike E, Muhammad A, Aliyu S, Abdullahi AS. 2013. Effects of fungal (*Lachnocladium* spp.) pretreatment on nutrient and antinutrient composition of corn cobs. Afr J Biochem Res 7: 210-214. DOI: 10.5897/AJBR2013.0715.

Pathak P, Bhardwaj NK, Singh AK. 2014. Production of crude cellulase and xylanase from *Trichoderma harzianum* PPDDN10 NFCCI-2925 and its application in photocopier waste paper recycling. Appl Biochem Biotechnol 172: 3776-3797. DOI: 10.1007/s12010-014-0758-9.

Peciulyte A, Anasontzis GE, Karlström K, Larsson PT, Olsson L. 2014. Morphology and enzyme production of *Trichoderma reesei* Rut C-30 are affected by the physical and structural characteristics of cellulosic substrates. Fungal Genet Biol 71: 64-72. DOI: 10.1016/j.fgb.2014.07.011.

Poeta P, Dias AA, Igrejas G, Silva V, Bezerra R, Nunes CS. 2018. Selection, engineering, and expression of microbial enzymes In: Nunes C, Kumar V, Steudler S, Werner A, Cheng J (eds). Enzymes in Human and Animal Nutrition: Principles and Perspectives. Elsevier Academic Press, United Kingdom. DOI: 10.1016/B978-0-12-805419-2.00001-0.

Sharma KK, Singh US. 2014. Cultural and morphological characterization of rhizospheric isolates of fungal antagonist *Trichoderma*. J Appl Nat Sci 6: 451-456. DOI: 10.31018/jans.v6i2.481.

Sharma K., Thakur A, Goyal A. 2019. Xylanases for food applications. In: Varjani B, Raveendran S (eds). Green Bio-process: Enzymes in Industrial Food Processing. Springer, Singapore. DOI: 10.1007/978-981-13-3263-0_7.

Soliman HM, Sherief A-DA, EL-Tanash AB. 2012. Production of xylanase by *Aspergillus niger* and *Trichoderma viride* using some agriculture residues. Intl J Agric Res 7: 46-57. DOI: 10.3923/ijar.2012.46.57.

Srinivasa N, Devi TP, Sudhirkumar S, Kamil D, Borah JL, Prabhakaran N. Bioefficacy of *Trichoderma* isolates against soil-borne pathogens. Afr J Microbiol Res 8: 2710-2723. DOI: 10.5897/AJMR2013.6588.

Steudler S, Werner A, Walther T. 2019. It is the mix that matters: substrate-specific enzyme production from filamentous fungi and bacteria through solid-state fermentation In: Steudler S, Werner A, Cheng J (eds). Solid State Fermentation Advances in Biochemical Engineering/Biotechnology. Springer, Switzerland. DOI: 10.1007/10_2019_85.

Tarayre C, Bauwens J, Brasseur C, Mattéotti C, Millet C, Guiot P A, Destain J, Vandenbol M, Portetelle D, De Pauw E, Haubruge E, Francis F, Thonart P. 2015. Isolation and cultivation of xylanolytic and cellulolytic *Sarocladium kiliense* and *Trichoderma virens* from the gut of the termite *Reticulitermes santonensis*. Environ Sci Pollut Res 22: 4369-4382. DOI: 10.1007/s11356-014-3681-2.

Van Soest PJ, Robertson JB, Lewis BA. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J Dairy Sci 74: 3583-3597. DOI: 10.3168/jds.S0022-0302(91)78551-2.

Vandenberghe LPS, Soccol CR, Pandey A, Lebeault J-M. 1999. Microbial production of citric acid. Braz Arch Biol Technol 42. DOI: 10.1590/S1516-89131999000300001.

Verma D, Satyanarayana T. 2012. Molecular approaches for ameliorating microbial xylanases. Bioresour Technol 117: 360-367. DOI: 10.1016/j.biortech.2012.04.034.

Xue Y, Cui X, Zhang Z, Zhou T, Gao R, Li Y, Ding X. 2020. Effect of β-endoxylanase and α-arabinofuranosidase enzymatic hydrolysis on nutritional and technological properties of wheat brans. Food Chem 302: 125332. DOI: 10.1016/j.foodchem.2019.125332.