Cellulase and Xylanase Production from Three Isolates of Indigenous Endophytic Fungi

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Abstract. Cellulases and hemicellulases have good potential to be used in energy production, in pulp, paper, textile industries, as well as in animal feed industries. Moreover, its utilization in food industries also cannot be ignored, among others, cellulase and xylanase roles in bakery, wine, and fruit and vegetables juice production. One of the potential enzyme source is endophytic fungi. Object of this study is to explore the potency of endophytic fungi isolated from medicinal plants as source of cellulolytic and xylanolytic enzymes. HL.47F.216 is endophytic fungi isolated from traditional medicinal plants ironwood tree was determined as xylanase producer. HL.51F.235 from pin-flower tree is cellulase producer, while CBN.6F.29 which produces both xylanase and cellulase is originated from Madagascar periwinkle. HL.47F.216 showed 2.5 cm in clear zone diameter and its xylanase activity was 0.262 U/mL with optimum condition pH 7 at 50°C. HL.51F.235 showed 2.4 cm clear zone diameter and 0.239 U/mL of cellulase activity at pH 5 and 70°C. CBN.6F.29 showed 2.8 cm and 0.394 U/mL (pH 5, 40°C) for its cellulase activity, while 2.3 cm and 0.439 U/mL (pH 8, 70°C) for its xylanase activity. Xylanase from HL.47F.216 and CBN.6F.29 showed low molecular masses of 20 kDa and 37-50 kDa, respectively. Molecular masses for cellulases from HL.51F.235 and CBN.6F.29 were 25 and 100 kDa for HL.51F.235 and 100 kDa for CBN.6F.29. Based on macroscopic and microscopic identification, fungal isolate CBN.6F.29 is a member of Class Coelomycetes, while HL.47F.216 was Acremonium sp. and HL.51F.235 was Aspergillus nigri.

Keywords: Bioprocess, Cellulase, Endophytic fungi, Xylanase

1. Introduction

Recent studies focus on cellulases and xylanases are highly increase in number. It is due to their high demand in worldwide markets for food and beverage industries. Cellulase decompose cellulose, a recalcitrant polymer formed from D-glucose residues linked by β-1,4-glycosidic bonds. On the other hand, xylan, a complex polysaccharide consisted of a backbone of xylose residues linked by β-1,4-glycosidic bonds is degraded by xylanase.

Cellulases are employed for their capability to increase the yield of potato pulp maceration, guava juice clarification, and tomato juice filtration (Ali 2003). Kuhad et al. (2011) emphasized their usefulness by mentioning their applications in food industry. These enzymes can release antioxidants from fruit and vegetable pomace, improve starch and protein extraction yields, modify the viscosity of fruit purees, control bitterness of citrus fruit, and take roles in texture and quality of bakery products improvement.

Beg et al. (2001) stated that xylanases are also remarkable in baking industries, increasing the specific bread volume. In biscuit making, it is recommended for making cream crackers lighter (Harris...
& Ramalingam 2010). Both cellulase and xylanase from more than one microorganism are better than when obtained from a single microorganism when applied to improve the extraction of olive oil (Bhat 2000).

Microorganisms are frequently used as source of enzymes because they can be cultured in large quantity but in a short time period. Moreover, microbial proteins are often more stable than enzymes from other sources, such as animal or plant origin (Archarya & Chaudhary 2012). Fungi are well-known as agents of decomposition of organic matter in general and of cellulosic substrate in particular (Jahangeer et al. 2005). Therefore, commercial enzymes that are on the market are often fungal enzymes, including cellulases and xylanase (Polizeli et al. 2005). Fungal cellulases can be produced in large amounts and often are less complex than bacterial cellulases. This makes it easier to clone the fungal genes and to produce the corresponding enzymes through recombination method for advanced up-scaling (Archarya & Chaudhary 2012).

Endophytic fungi from root, rod, or leaves of plants have potential for cellulase and xylanase production in industries since they have adapted in cellulosic and hemicellulosic materials (Robl et al. 2013, Sunitha et al. 2013). They spend the entire or part of their life cycle colonizing intercellular and/or intracellular spaces inside the healthy tissues of the host plants, typically causing no apparent symptoms of disease (Ravindran et al. 2010). Foliar fungi from medicinal plants even may play a role in the function of leaves as traditional medicines (Maria et al. 2005). This information emphasizes the great potency of medicinal plants endophytes as enzymes producers for food industry (Patil et al. 2015). The aim of the present study is to determine cellulase and xylanase secretion from indigenous endophytic fungi isolated from medicinal plants in Indonesia.

2. Materials and Methods

2.1 Microorganisms

Unidentified endophytic fungi belonging to the Research Center of Biotechnology, Indonesia Institute of Science, were used. CBN.6F.29 was isolated from leaf of Madagascar periwinkle (Catharanthus roseus) in Cibinong. HL.47F.216 was isolated from the middle rod of ironwood tree (Memecylon excelsum Blume), while HL.51F.235 originated from leaf of pin-flower tree (Glochidion borneense). Both were sampled from Mount Halimun National Park.

2.2 Enzyme Production

Pre-culture of the isolates was carried out in Potato Dextrose Broth for 72 h with shaking at 110-120 rpm. Ten percent (10%) of each pre-culture along with potato extract (10%) and sugarcane bagasse (2.5%) was employed as a culture of the isolates. The latter was incubated for 7 d with shaking at 110-120 rpm at room temperature (25-28°C). To obtain crude enzyme extract, twice centrifugation at 13,000 rpm and 4°C for 5 min each was conducted. Subsequently, filtration with filter paper was carried out if required.

2.3 Screening of Cellulase and Xylanase Activity

Cellulase and xylanase activities were first qualitatively observed by the Congo red assay. Carboxymethyl cellulose (CMC) and xylan from beechwood were used as substrate for cellulase and xylanase activity determination, respectively. Crude enzyme was spotted on double layer agar pH 6 supplemented with 0.5% substrate. Incubation for 3 d was conducted at 37°C prior to the Congo red assay. To get better visualization of clear zones, acetic acid (5%) was then flooded to the plates. Each sample was subjected to protein concentration determination at 280 nm and protein visualisation using SDS-PAGE and zymogram analysis (Laemmli 1970). Polyacrylamide gels 10% were employed on vertical electrophoresis for 1.5-2 h at 200 W 40 mA 100 V. Staining in SDS-PAGE analysis was done by 0.02% Coomassie Brilliant Blue G-250 and proteins marker (Bio-Rad, Precision Plus Protein Standards Dual Color) were used as an indicator of protein molecular mass. Blue bands showed the protein profile. Gels for zymogram analysis were soaked in 2.5% Triton X-100 for 30 min at 25 rpm, followed by rinsing with milliQ water for 5 min, which was done twice. The gel was then soaked in 50
mM buffer phosphate pH 6 for an hour at 45°C 50 rpm. The gel was rinsed with milliQ water and stained with 0.1% Congo red for 30 min. 1 M NaCl was employed in gel destaining, while 5% acetic acid was used for clearer visualization. Protein showed as unstained clear bands against a dark-purple background.

2.4 Optimization of cellulase and xylanase reaction

Activities of xylanase and cellulase were quantified by incubating 250 μL xylan or CMC (0.5%), respectively, with 250 μL enzyme sample, followed by the DNS assay for determination of the reducing sugars (Miller 1959). The pH value was optimized in the range of pH 4-10 using citrate (pH 4 and 5), phosphate (pH 6 and 7), and glycine (pH 8-10) buffer as substrate solvent. Optimization of temperature then was conducted in the range of 30°C-100°C.

2.5 Fungal identification

Morphological identification of fungal isolates was conducted macroscopically and microscopically by Indonesia Culture Collection (Barnett & Hunter 1998; Domsch et al. 1980; Samson et al. 1995). Fungal isolates were grown on Potato Dextrose Agar and identified using light microscope Olympus BX53 with bright field exposure and magnification scale up to 1000X using immersion oil. Camera Olympus DP 26 which connected to computer was used for microscopic documentation.

3. Result and Discussion

3.1 Screening of cellulase and xylanase activities

The Congo red assay showed that HL.47F.216 and HL.51F.235 have different abilities and enzyme profile. The latter was shown as a CMC degrader, while HL.47F.216 is able to utilize xylan. HL.47F.216 formed a 2.5 cm clear zone in diameter and HL.51F.235 formed a clear zone with 2.4 cm diameter, indicating their xylanolytic and cellulolytic ability, respectively. CBN.6F.29 was shown to have both xylanolytic and cellulolytic features. Its xylanase formed a bigger clear zone than HL.47F.216, 2.8 cm. However, its clear zone diameter on CMC plate did not differ much to the one of HL.51F.235, 2.3 cm (Figure 1). The initial screening showed that the endophytes have a massive ability to degrade cellulose and hemicellulose since they produced clear zones of >2 cm. These abilities may be gained due to the adaptation of the fungi to their habitat, which is in a set of lignocellulosic materials in the body of plants (Bhagobaty & Joshi 2012).

Results of zymogram analysis as depicted in Figure 2, show that 25 kDa and 50 kDa proteins are responsible for CMC decomposition by HL.51F.235. On the other hand, a higher molecular mass was shown for the cellulase from CBN.6F.29, approximately 100 kDa. The enzyme responsible for the
decomposition of xylan appeared on the analysis at 20 kDa in the crude enzyme sample of HL.47F.216, while it shows a mass between 37 and 50 kDa for CBN.6F.29. SDS-PAGE analysis showed that protein pattern from these samples have distinct differences between substrates, indicating true endocellulases or xylanases.

![Figure 2](image.png)

Figure 2. SDS-PAGE and zymogram analysis on 10% polyacrylamide gel. Blue bands in SDS-PAGE gel showed profile of protein from the sample. Unstained bands indicated particular protein role in CMC or xylan degradation. (a) cellulase, M: Marker; 1: HL.51F.235; 2: CBN.6F.29 (b) xylanase. M: Marker; 1: HL.47F.216; 2: CBN.6F.29.

The molecular masses of the xylanases or cellulases identified in this study are in good agreement with prior researches. The molecular mass of HL.51F.235 cellulase is similar to those from *Trichoderma reesei* and *Phanerochaete* reported in Nayebyazdi *et al.* (2012) ranging from approximately 25 to 50 kDa. Ncube (2013) also reported a molecular mass of 20-43 kDa for *Aspergillus niger* cellulases, while Ritter *et al.* (2013) and Li *et al.* (2011) stated in a review that the molecular weight of fungal cellulase can span a wide range (30-250 kDa). The present study corresponds to the latter as CBN.6F.29 cellulase was detected at approximately 100 kDa.

The molecular mass of xylanases from the endophyte isolates was at 20-50 kDa, in agreement to previous studies (Polizeli *et al.* 2005). *Aspergillus aculeatus* F50 xylanases were 18 kDa, 26 kDa, and 52 kDa (Fujimoto *et al.* 1995), *Hypocrea lixii* xylanase was 29 kDa (Sakthiselvan *et al.* 2014), and *Neocallimastix frontalis* xylanases were 45 kDa and 70 kDa (de Segura & Favre, 1993) in mass. A xylanase with low molecular weight (19 kDa) was also found in *Aspergillus fumigatus* (Silva *et al.* 1999).

### 3.2 Optimization of pH value and temperature

Enzyme activity assays using the DNS method were conducted for 15 min reaction time. These data (Figure 3) showed that the optimal pH for cellulase activity was 5 for crude enzyme samples from both isolates, and declined as the pH value rose. The patterns of optimal temperature of cellulase activity from HL.51F.235 and CBN.6F.29 were quite different. HL.51F.235 was rather high, 70°C, whereas cellulase from CBN.6F.29 reacts optimally at moderate temperature, 40°C. On the other hand, xylanase from HL.47F.216 and CBN.6F.29 have higher optimal pH. Moreover, they still have considerable activities at pH 10. Xylanase from CBN.6F.29 was alkaline as its optimal pH was 8 at 70°C. Xylanase from HL.47F.216 was optimal at pH 7 at 50°C. In general, HL.47F.216 had a lower xylanase activity compared to CBN.6F.29.
A summary of enzymes activities of the endophytes presented in Table 1. Cellulase activity of HL.51F.235 and CBN.6F.29 cultivated on sugarcane bagasse were 0.239±0.0061 U/mL and 0.394±0.0224 U/mL, respectively. Samples from HL.47F.216 have an activity of 0.262±0.0172 U/mL, while CBN.6F.29 shows 0.439±0.0003 U/mL for xylanase.

Table 1. Enzymes activities and properties determined for crude culture supernatants from the three endophytic fungi cultivated on media containing sugarcane bagasse

| No | Isolate   | Optimal Condition | Enzyme Activity (U/mL) |
|----|-----------|-------------------|------------------------|
|    |           | pH    | Temperature | Cellulase (CMCase) | Xylanase |
| 1  | HL.47F.216| 7     | 50 °C       | -                  | 0.262±0.0172 |
| 2  | HL.51F.235| 5     | 70 °C       | 0.239±0.0061       | -        |
| 3  | CBN.6F.29 | 5     | 40 °C       | 0.394±0.0224       | -        |
|    |           | 8     | 70 °C       | -                  | 0.439±0.0003 |

3.3 Fungal isolates identification

Macroscopically, fungal isolate CBN.6F.29 looked like cotton on PDA media. It was white yet no color on reversionary colony. Its hyphae was examined under the microscope and found that it was transparent (hyaline), septe (monocytic) with no clamp connection structure, and 3-5 μm in width. This fungal isolate did not formed reproduction structure neither sexual nor asexual during sterile incubation (Figure 4). Based on these information, CBN.6F.29 was identified as member of Class Coelomycetes. Taxon was showed on Table 2.

Coelomycetes is a general term for asexual fungi and it has three genera, Phyllosticta, Phomopsis, and Phloeospora. Coelomycetes occupy many ecological niches and may be pathogens of terrestrial or aquatic plants, endophytes, or saprobes. Some endophytic species may be latent pathogens, existing as symptomless endophytes, but expressing themselves as pathogens once the host defense system weaken. This Class of fungi are common in tropical and temperate regions. It is used as biological control agents, bioremediator, and for pharmaceutical uses (Wijayawardene et al. 2012).
Center part of fungal isolate HL.47F.216 was pale pink cotton-like, rather wet, and slimy. Its reversionary colony was found to be creamy white. Hyphae was found transparent, septate (monocytic), 3-5 μm in diameter with no clamp connection structure. It had single/ramified conidiophore. Fialid formed at substrate and pustules mycelium. Its huddle conidia formed slimy head conidia. Conidia was short oval and lengthwise sized 3-5 μm x 1-2 μm. Clamydiospore was not found during incubation on PDA. *Acremonium* sp. was considered to be alike with HL.47F.216 based on the information investigated.

Table 2. Taxon of fungal isolates identified macroscopically and microscopically

| Takson      | CBN.6F.29 | HL.47F.216 | HL.51F.235 |
|-------------|-----------|------------|------------|
| Kingdom     | Fungi     | Fungi      | Fungi      |
| Division    | Eumycota  | Eumycota   | Eumycota   |
| Subdivision | Deuteromycotina | Ascomycotina | Ascomycotina |
| Class       | Coelomycetes | Ascomycetes | Ascomycetes |
| Order       | -          | Hypocreales | Eurotiales |
| Family      | -          | Hypocreaceae | Trichocomaceae |
| Genus       | -          | *Acremonium* | *Aspergillus* |
| Subgenus    | -          | *Acremonium* sp. | *Aspergillus* section Nigri |

Figure 5. (A) Macroscopic appearance of fungal isolate HL.47F.216, (B) anamorphic structure of conidiophore, fialid, and group of conidia (magnification 1000X)

Figure 6 showed that fungal isolate HL.51F.235 was like black powder and its reversionary colony was no color. It had transparent hyphae, septate (monocytic), 4-6 μm in width, and clamp connection structure was not found. Its conidiophore was in color (hyaline), had smooth wall and its rounded ends formed vesicle. Vesicle was round to semi-round and 33-55 μm in diameter. Fialid was directly formed at vesicle or at metula (biseriate). Sclerotia structure was not found during incubation.
on PDA. Fungal isolate HL.51F.235 was determined as *Aspergillus* section *nigri* with taxon showed on Table 2.

![macroscopic appearance of fungal isolate HL.51F.235](image1)

![anamorphic structure of conidia](image2)

![stipe conidiophore and conidium head](image3)

![vesicle and conidia](image4)

Figure 6 (A) macroscopic appearance of fungal isolate HL.51F.235, B) Anamorphic structure of conidia (magnification 100X) (C) Stipe conidiophore and conidium head (magnification 400X) (D) vesicle and conidia (magnification 1000X)

4. Conclusions
Three isolated endophytic fungi, HL.47F.216, HL.51F.235, and CBN.6F.29 could produce cellulase and xylanase. Further detail investigations are needed to improve the activities and to make their production economically feasible. Besides, several biochemical and toxicological tests are required to ensure that the endophytes from the medicinal plants are food-grade fungi. Hence, morphological, biochemical, and genetic identification along with finding the efficient conditions in enzymes production are going to be the future aims of the study.

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References
[1] Acharya S, Chaudhary A. 2012. Bioprospecting thermophiles for cellulase production: A review. *Braz. J. Microbiol.* 844-856.
[2] Ali SG. 2003. Biotechnological application of alkaline cellulase in food technology. *Ass. University Bull Environ Res* 6: 117-124.
[3] Barnett HL, Hunter BB. 1998. Illustrated genera of imperfect fungi 4th ed. New York (USA): Prentice Hall, Inc.
[4] Beg QK, Kapoor M, Mahajan L, Hoondal G. S. 2001. Microbial xylanases and their industrial applications: a review. *Appl Microbiol Biot* 56: 326-338.
[5] Bhagobaty RK, Joshi SR. 2012. Enzymatic activity of endophytic fungi on five medicinal plant species of the pristine sacred forests of Meghalaya, India. *Biotechmol Biopro Eng* 17: 33-40.
[6] Bhat MK. 2000. Cellulases and related enzymes in biotechnology. *BiotechAdv* 18: 355-383.
[7] De Segura BG, Fevre M. 1993. Purification and characterization of two 1,4-β-xylan endohydrolases from the rumen fungi *Neocallimastix frontalis*. *Appl Environ Microbiol* 59: 3654-3660.
[8] Domsch KH, Gams W, Anderson TH. 1980. Compendium of soil fungi. Vol 1. London (UK):
Academic Press.

[10] Fujimoto H, Oor T, Wang S, Takizawa T, Hidaka H, Murao S, Arai M. 1995. Purification and properties of three xylanases from Aspergillus aculeatus. Biosci Biotech Biochem 59: 538-540.

[12] Harris AD, Ramalingam C. 2010. Xylanases and its application in food industry: A review. J Exp Sci 1: 1-11.

[13] Jahangeer S, Khan N, Jahangeer S, Sohail M, Shahzad S, Ahmad A, Khan SA. 2005. Screening and characterization of fungal cellulases isolated from the native environmental source. Pak J Bot 37: 739-748.

[14] Kuhad RC, Gupta R, Singh A. 2011. Microbial cellulases and their industrial applications. Enz Res, 1-10.

[15] Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227(5259): 680-685.

[16] Li D, Li A, Papageorgiou A. 2011. Cellulases from thermophilic fungi: Recent insights and biotechnological potential. Enz Res, 1-9.

[17] Maria GL, Sridhar KR, Raviraja NS. 2005. Antimicrobial and enzyme activity of mangrove endophytic fungi of southwest coast of India. J Agr Tech 1: 67-80.

[18] Miller GL. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31: 426-428.

[19] Nayebyazdi N, Salary M, Ghanbary MAT, Ghorbany M, Bahmanyar M. 2012. Investigation of cellulase activity in some soil borne fungi isolated from agricultural soils. Ann Biol Res 3: 5705-5713.

[20] Ncube T. 2013. Development of a fungal cellulolytic enzyme combination for use in bioethanol production using Hyparrhenia spp. as a source of fermentable sugars. PhD thesis. Limpopo: University of Limpopo.

[21] Patil MG, Pagare J, Patil SN, Sidhu AK. 2015. Extracellular enzymatic activities of endophytic fungi isolated from various medicinal plants. Int J Curr Microbiol Appl Sci 4: 1035-1042.

[22] Polizeli MLTM, Rizzati ACS, Monti R, Terenzi HF, Jorge JA, Amorim DS. 2005. Xylanases from fungi: properties and industrial applications. Appl Microb Biot 67: 577-591.

[23] Ravindran C, Naveenan T, Varatharajan G. 2010. Optimisation of alkaline cellulase production from marine derived fungi, Chaetomium sp., using agricultural and industrial wastes as substrates. Bot Mar 53: 275-282.

[24] Ritter CET, Camassola M, Zampieri D, Silveira MM, Dillon AJP. 2013. Cellulase and xylanase production by Penicillium echinulatum in submerged media containing cellulose amended with sorbitol. Enz Res, 1-9.

[25] Robi D, Delabona PS, Mergel CM, Rojas JD, Costa PS, Pimentel IC, Vicente VA, Pradella JGC, Padilla G. 2013. The capability of endophytic fungi for production of hemicellulases and related enzymes. BMC Biot 13: 94.

[26] Sakthiselvan P, Naveena B, Partha N. 2014. Molecular characterization of a xylanase-producing fungi isolated from fouled soil. Braz J Microbiol 45: 1293-1302.

[27] Samson RA, Hoekstra ES, Frisvad JC, Filtenborg O. 1995. Introduction to food borne fungi. 4th ed. Netherlands: Ponsen & Looyen.

[28] Silva CHC, Puls J, de Sousa MV, Filho EXF. 1999. Purification and characterization of a low molecular weight xylanase from solid-state cultures of Aspergillus fumigatus. Rev Microbiol 30: 114-119.

[29] Sunitha VH, Devi DN, Srinivas C. 2013. W J Agr Sci 9: 1-9.

[30] Tong CC, Cole AL, Shepherd MG. 1980. Biochem J 191: 83-94.

[31] Wijayawardene NN, McKenzie EHC, Chukateirote E, Wang Y, Hyde KD. 2012. Coelomycetes. Crypt Mycol 33: 215-244.