Cellulolytic Fungi Isolation and Identification from Fermetoge, Cattle Feed Composed of Fermented Water Hyacinth (Eichhornia crassipes) And Corn (Zea mays) Cob

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Abstract. Fermetoge is fermented feed produced from fermentation of water hyacinth and corn cob mixture, commonly used as cattle feed in East Java, Indonesia. Fermetoge is composed of these cellulose-contained materials easily degraded by cellulase enzymes produced by fungi during fermentation. This study was aimed to isolate fungi from Fermetoge and screen their ability in degrading cellulose. Ten fungi isolates were found from fermetoge, three out of which indicated high cellulolytic activity. The three of them were identified based on partial ITS 1 and ITS 4 gene sequencing as Rhizopus stolonifer, Aspergillus fumigatus, and Penicillium sp., with similarity level of 95%, 100%, and 89% respectively. Isolation and identification of fungi from fermetoge can give insight into cellulose-degrading fungi species to be studied for further developing starter culture and improving the quality of starter-base fermented feed.

Key word: Fermetoge, cellulolytic activity, cellulose-degrading fungi

Introduction

Water hyacinth grows rapidly in water polluted by organic materials [1]. Up until now, water hyacinth is assumed as invasive and disrupting aquatic weeds. Its rapid growth in the surface of aquatic body means it is able to alter organism diversity and endanger other organisms in the water [2]. Various efforts had been conducted to suppress their growth, including nutrient reduction in aquatic body [2], manual removal of plants, and addition of 2,4-D or glyphosate [3] and paraquat [4].

Other effort performed to reduce the population of water hyacinth is to process it into cattle-feed due to its high level of protein, at around 11.87% to 14.28 % [5], high content of calcium and phosphor, and its ability to stimulate milk production when combined into feed at suitable concentration [6]. Other study found that water hyacinth contained dry materials (8.7-9.3 g/100g), crude proteins (10.1-11.2 g/100g), crude fibers (26.1-27.4 g/100g), nitrogen-free extracts (47.2-50.2 g/100g), ether extracts (1.1-1.8 g/100g), and total ash (12.3 to 12.4 g/100g), with metabolism energy up to 1999.7-2054.1 kcal/kg [7]. Previous studies had utilized water hyacinth as duck [8][9] and Cyprinus carpio feed [10].

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Other less utilized waste materials but potentially able to be used as cattle feed is corncob. Lignocellulose content of corncob was found to consist of 45-55% cellulose, 25-35% hemicellulose, and 20-30% lignin, which cannot be digested by pig digestive enzymes [11]. Corncob was also contained 5.6% protein, higher than that of rice straw (4.9%). Corncob had been previously processed to be pig, chicken, goat [12], buffalo [13][14], fish [15], and various kinds of ruminant feed [16].

Fermetoge is cattle feed made of fermented water hyacinth and corncob which have several advantages over regular feed, such as promoting cattle digestibility and nutrient absorbptivity level, and helping to balance rumen microflora [17]. Fermented feed could also lower pathogenic microbe growth populated digestive tract by helping stomach to reach more acidic pH, thus it can kill microbes carried in by food easier [17]. Administration of fermented feed for chicken not only could increase body weight and strengthen egg-shell without reduction in egg productivity, but also induce more aggressive behaviour [18]. Cellulase enzymes secreted by various fungi in fermetoge function to degrade cellulose available in starter culture from cellulosic ingredients, including plant material or agricultural waste, which possibly affect to the fermented feed quality.

Cellulase is able to be applied as biocatalyst in composting organic waste, for example agricultural waste such as corncob, aquatic weeds such as water hyacinth, and also in processing of bioethanol from cellulose-containing materials [19]. In the current era, fermented feed is developed especially in relation to utilize agricultural waste for raw ingredients of quality fermented feed. However cellulose degradation from agricultural waste and aquatic weed is still complicated, due to complex structure of cellulose composing plant cell wall. Degradation of cell wall cellulose can be conducted by enzymatic reaction using cellulase enzymes, such as cellulase R-10, Y-23 pektiolase, pectinase, macerozyme, and hemicellulase [20]. However, the use of commercial enzyme in industrial or agricultural processes is inefficient because of its high cost. The alternative is to use cellulase produced from cellulolytic fungi. Fungal cellulase enzymes is easy to develop because it is produced by cellulolytic fungi, which can be cultivated via cell or hyphae culture. Thus, the cost of using fungal cellulase can be lowered compared to commercial cellulase.

Based on the elaboration above, identification of cellulase-producing fungi and other microbe species therefore became important for screening their potential application in biotechnological industry. The current study was designed for isolating cellulase-producing fungi from fermetoge and identifying fungi species by using partial ITS 1 and ITS 4 gene amplification and sequencing.

Materials and Methods

1.1. Source Preparation and Fungi Isolation
Water hyacinth was collected from a river previously screened for safe water quality and heavy metal level, while corncob was obtained from rice mill tested safe from dangerous contaminant. As initial fungi sources, production of fermented feed was named fermetoge with ingredients mixture of water hyacinth and corncob at ratio 1:1. This feed is made by following steps; cutting, drying, steaming, and finally incubating materials to let fermentation occurred naturally [21]. During fermentation, fungi was isolated daily by taking 30 g materials randomly, then materials were suspended in sterile distilled water, before being filtered and cultured using pour plate method. Fungi was cultured in PDA (Potato Dextrose Agar for Microbiology Merck) medium and incubated at 37°C for 3-7 days.

1.2. Purification, Characterization and Identification isolated Fungi
Culture grown was then purified. Characterization and identification of purified fungi isolates was performed using macroscopic and microscopic characters observation. Species was confirmed using ITS1 and ITS 4 partial sequence analysis.

1.3. Fungi Cellulolytic Activity Test
Fungi cellulolytic activity was evaluated based on halo zone formation appeared in growth medium colored with 2% Congo Red. Medium used in cellulolytic test was CMC agar medium (1000 ml distilled
water, 1 g NH4H2PO4, 0.2 g KCl, 1 g MgSO4.7H2O, 1 g yeast extract, 26 g CMC, and 3 g agar). Halo zone indicated positive cellulolytic activity of fungi isolate. Isolate with large halo zone could be potentially applied in cellulosic-material degrading starter formula.

1.4. Identification of Cellulolytic Fungi Isolates
Isolation of genomic DNA from purified cellulolytic fungi subculture was performed using QIAMP DNA Mini Kit (Qiagen) by following manufacturer’s guideline. DNA Quantification was conducted using Maestronanopro (Maestrogen). Amplification of ITS 1 and ITS 4 genes of isolated fungi was performed in accord with standardized method with few modifications. Universal primer used to amplify ITS1 gene had sequence of 5'-TCCGTAGGTGAACCTGCGG-3', while primer for ITS4 amplification was 5'- CTCGGCTTATTGATAGTGC-3'(Bioneer). PCR reaction mix was composed of 12.5µL 2x Taq Master Mix (Bioron), 5pmol/µL of respective primer (1µL), 6.5 µL nuclease-free water, and 5 µL template DNA. Thermocycler (Eppendorf Master cycler Gradient) was set at following cycle; initial or pre-denaturation at 95°C for 5 min, denaturation at 95°C for 60 s, annealing at 50°C for 60 s, and finally extension at 72°C for 60 s for 35 cycles.

Amplified gene was visualized using ethidium bromide-stained 1.0% (w/v) Agarose gel D-1 Low EED (Pronadise, Spain) in TAE buffer (Bio Basic, Canada) under UV trans-illuminator (Bio-Rad). Size of DNA fragments were extrapolated from 1-kb DNA ladder (Norgen Biotek Corporation, Canada). Resulting gene were sequenced in Bioneer (Korea). Sequences of partial ITS 1 and ITS 2 genes were read using ABI 3730XL DNA Analyzer software and aligned in Basic Local Alignment Search Tool (BLAST) of National Center for Biotechnology Information (NCBI) to compare the sequences to existing sequences in GeneBank database and to identify fungi species based on similarity percentage.

3. Results and Discussion
Ten fungi colonies were successfully isolated from fermetoge, which were then screened for their cellulolytic activity on CMC agar medium. Three fungi isolates, coded F2, F3, and F4, had high cellulolytic activity, based on their ability to form halo zone as visualized using Congo red staining. ITS1 and ITS4 partial gene amplification was performed to the three isolates using fungi-specific universal forward and reverse primers. Visualization of amplified DNA of M (positive control), K- (negative control), and all three fungi isolates were showed in Figure 1.

DNA sequencing was then performed to partial ITS1 and ITS4 of F2, F3, and F4. Resulting sequences were aligned against BLAST (NCBI) database entries, which revealed that F2 had 95% similarity to Rhizopus stolonifer, F3 had 100% similarity to Aspergillus fumigatus, while F4 was 89% similar to Penicillium sp. (Table 1).

![Figure 1](image-url) - Visualization of isolated fungi amplified gene (F2, F3, F4) with comparison to marker (M) and negative control (K-).
Table 1. Sequence homology of the three fungi isolates with the GeneBank database

| Isolate Code | Species                  | Homology (%) | Accession Number |
|--------------|--------------------------|--------------|------------------|
| F2           | Rhizopus stolonifer      | 95           | DQ767605.1       |
| F3           | Aspergillus fumigatus    | 100          | JN252124.1       |
| F4           | Penicillium sp.          | 89           | LC109278.1       |

The three fungi species with high cellulolytic activity were isolated from fermetoge in the current study. They have been generally implicated in various types of cellulose fermentation, such as the ones occurring in highly-cellulosic *Chlorella zofingiensis* [22]. Cellulolytic activity of *Penicillium* had been studied in extent previously, for example to degrade *salak* leaf waste and shown to produce a number of cellulase enzymes, such as endoglucanase, cellobiohydrolase, and β-glucosidase [23]. *Penicillium brasillianum*, a species of *Penicillium* genus, as well as *Trichoderma reesei*, were found to not only have cellulolytic activity, but also xylanolytic activity.

Based on previous study, *Aspergillus fumigatus*, which was found in the current study, could synthesize exoglucanase (EXG; EC: 3.2.1.91), endoglucanase (EG; EC: 3.2.1.4), CMCase, β-glucosidase (BG; EC: 3.2.1.21) and xylanase (Xy; EC: 3.2.1.8) using rice straw and wheat plant waste mixture as fermentation substrate in solid-state fermentation process [24]. These enzymes belong to cellulase group; thus *A. fumigatus* can be a potential fungus to be applied in fermentation of water hyacinth and corncob mixture.

*Rhizopus stolonifer* in another study was able to degrade 70% starch and 81% cellulose found in cassava waste within 8 days [25], showing that this fungus has high cellulolytic activity. In addition to starch and cellulose, cassava waste also contained reduced sugars and proteins. During 8 days of fermentation, protein content in cassava waste rose up to 89.4 mg/g. Other than cellulase, *Rhizopus* fungi are also able to produce carbohydrate-degrading enzymes. *Rhizopus stolonifer* had been previously applied for cellulase production in solid state fermentation of *Artocarpus heterophyllus* pulp/wood pulp, which was found to have higher enzyme activity and enzyme level compared to *Penicillium*.

4. Conclusion

Three fungi isolates from fermetoge were found to possess high cellulose-degrading enzyme activity, possibly contributing to the quality of this ruminant fermented feed. The fungi able to degrade cellulose were identified through macroscopic and microscopic characters and confirmed by partial ITS1 and ITS4 gene sequence as *Rhizopus stolonifer*, *Aspergillus fumigatus*, and *Penicillium sp*. Further characterization of these fungi species may have advantage for the development of starter cultures to enhance quality of fermented feed product. The development of starter culture is quite necessary to be potentially applied in commercial-level production of animal fermented feed. Cellulose-degrading fungi can also be further investigated for their potential to produce cellulase enzymes that possibly be used in industrial-level application.

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6. References
[1] Ndimele, P. & Jimoh, A. 2011. Water hyacinth (Eichhorniacrassipes [Mart.] Solms.) in phyto remediation of heavy metal polluted water of Ologe Lagoon, Lagos, Nigeria. Research journal of Environmental Sciences, 5(5): 424-433. DOI: 10.3923/rjes.2011.424.433.

[2] Gichuki, J., Omondi, R., Boera, P., Okorut, T., Matano, A. S., Jembe, T. & Ofulla, A. 2012. Water hyacinth (Eichhorniacrassipes [Mart.] solms-laubach dynamics and succession in the Nyanya Gulf of Lake Victoria (East Africa): implications for water quality and biodiversity conservation. The Scientific World Journal, 10: 11-20. DOI: 10.1100/2012/106429.

[3] Labrada, R., Caseley, J. C. & Parker, C. 1994. Weed Management for Developing Countries. FAO. New York.

[4] Jian-jun, C., Yi, D. & Qi-jia, Z. 2006. Invasión and control of water hyacinth (Eichhorniacrassipes) in China. J Zhejiang Univ SCIENCE B, 7(8): 623-626. DOI: 10.1631/jzus.2006.B0623.

[5] Mako, A. A., Babayemi, O. J. & Akinsoyinu, A. O. 2011. An evaluation of nutritive value of water hyacinth (Eichhorniacrassipesmart. solms-laubach) harvested from different water sources as animal feed. Livestock Research for Rural Development, 23: 106-110. http://www.lrrd.org/lrrd23/5/mako23106.htm (accessed on 23 April 2017).

[6] Kumar, A., Sharma, P. C., Kumar, A. & Negi, V. 2011. A study on phenotypic traits of Candida species isolated from blood stream infections and their in vitro susceptibility to fluconazole. Al Am een J Med Sci, 7(1):83-91.

[7] Hassain, Md. E., Sikder, H., Kabir, Md. H. & Sarma, S. M. 2015. Nutritive value of water hyacinth (Eichhorniacrassipes). Journal of Animal and Feed Research, 5(2): 40-44. http://www.science-line.com/index.

[8] Lu, J., Fu, Z. & Yin, Z. 2008. Performance of water hyacinth (Eichhorniacrassipes) system in the treatment of wastewater from a duck farm and the effects of using water hyacinth as duck feed. Journal of Environmental Sciences, 20(5): 513-519. DOI: 10.1016/S1001-0742(08)62088-4.

[9] Mangisah, I., Sukamto, B. & Nasution, M. H. 2009. Implementation of fermented ecenggondok in duck ration. Journal of the Indonesian Tropical Animal Agriculture, 34 (2): 127-133.

[10] Mohapatra, S. B. 2015. Utilization of water hyacinth (Eichhorniacrassipes) meal as partial fish protein replacement in the diet of Cyprinuscarnio Fry. European Journal of Experimental Biology, 5(5): 31-36.

[11] Kanengoni, A. T., Chimonyo, M., Ndima, B. K. & Dzama, K. 2015. Potential of using maize cobs in pig diets — a review. Asian Australas. J. Anim. Sci., 28(12): 1669-1679. DOI: 10.5713/ajas.15.0053.

[12] Sarian, Z. B. 2016. Corn cobs converted into nutritious animal feed. http://www.zacsarian.com/category/agri-ideas (accessed 30 October 2017).

[13] Wanapat, M., Pilachai, K., Wanapat, M., Pakdee, P. & Cherddhong, A. 2016. Effect of ground corn cobs replacement for cassava chip on fermentation and urinary derivatives in swamp buffaloes. Asian J. Anim. Sci., 25(8): 1124-1131. DOI: 10.5713/ajas.2012.12109.

[14] Wachirapakorn, C., Pilachai, K., Wanapat, M., Pakdee, P. & Cherddhong, A. 2016. Effect of ground corn cobs as a fiber source in total mixed ration on feed intake, milk yield and milk composition in tropical lactating crossbred holsteincows. Animal Nutrition 2: 334-338. DOI: 10.1016/j.aninu.2016.08.007.

[15] Rostika, R. & Safitri, R. 2012. Influence of fish feed corn-cob was fermented By Trichoderma sp., Aspergillus sp., Rhizopusoligosporusto the rate of growth of java barb (PuntiusGonionitus). APCBEE Procedia, 2: 148-152.

[16] Lardy, G. & Anderson, V. 2009. Alternative Feeds for Ruminant. NDSU.Dakota.

[17] Missotten, A. M., Michiels, J., Degroote, J., & De Sme S. 2015. Fermented Liquid Feed for Pigs: An Ancient Technique for The Future. Journal of Animal Science and Biotechnology, 6: 4-13.

[18] Engberg, R. M., Hammersh, M., Johansen, N. F., Abousekken, M. S., Steenfeldt, S. & Jensen, B. B. 2009. Fermented feed for laying hens: effects on egg production, egg quality, plumage condition and composition and activity of the intestinal microflora. Journal British Poultry Science, 50(2): 228-239. DOI: 10.1080/00071660902736722.
[19] Hidayanti, A.K. 2011. Isolasi bakteriamolitik dari Chlorophyta (Amylolytic bacteria isolation from Chlorophyta). Thesis, (S.Si.). Fakultas Biologi UGM. Yogyakarta.

[20] Sukmadjaja, D.N., Sunarlim, E.G., Lestari, I., Roostika, & Suhartini, T. 2007. Teknik isolasi and kultur protoplast tanaman padi (Isolation technique and protoplast culture of rice plant). Jurnal Agrobiogen, 3(2): 60–65.

[21] Fitrihidajati, H., Ratnasari, E., Isnawati, Soeparno, G. 2015. Quality of fermentation result of ruminant feed production made of water hyacinth (Eichorniacrassipes), Journal of Biosantifika, 7 (1): 62-67.

[22] Janatunaim, R.Z., Hamid, R.M., Christy, G. P., Purwestri, Y. A. & Tunjung, W. A. S. 2015. Identification of BSA B1 bacteria and its potency of purified cellulase to hydrolyze Chlorella zofingiensis. Indonesian Journal of Biotechnology, 20(1): 77-87.

[23] Sari, S. L. A., Setyaningsih, R., & Wibowo, N.F.A. 2017. Isolation and Screening of Cellulolytic Fungi from Salacca zalacca Leaf Litter. Biodiversitas, 18(3):1282-1288.

[24] Sherief, A. A., El-Tanash, A. B., & Atia, N. 2010. Cellulase Production by Aspergillus fumigatus Grown on Mixed Substrate of Rice Straw and Wheat Bran. Research Journal of Microbiology, 5 (3): 199-211.

[25] Pothiraj, C., Kanmani, P., & Balaji, P. 2006. Bioconversion of Lignocellulose Materials. Mycobiology, 34(4): 159-165.