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Full Length Article

Detection, Identification and Evaluation of Cellulase Enzyme Activity of Cellulolytic Fungi from Pliek U as Cellulose Hydrolysis Agent in Feed

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Abstract

Pliek U is a fermented product from coconuts which has been widely used for generations by the people of Aceh (Indonesia) as their typical food. Cellulolytic fungi are a group of fungi that are capable of producing cellulase enzymes which play a role in the fields of animal science, fisheries and pharmaceuticals, especially in hydrolyzing cellulose in feed. This study aimed to detect, identify and evaluate the cellulase enzyme activity of cellulolytic fungi from Pliek U as cellulose hydrolysis agent in feed. Parameters observed were macroscopic morphology (colony shape, colony size, colony elevation, colony margins, and colony color), identification of fungi from Pliek U and qualitative test of cellulolytic enzyme activity. The results obtained were nine isolates of cellulolytic fungi, which were able to hydrolyze cellulose qualitatively (0.87–4.11 cm). Two isolates came from the same strain, one isolate did not grow optimally, so it could not be identified. The identified fungal strains included *Nigrospora* spp., *Geotrichum* spp., *Aspergillus niger*, *Rhizoctonia solani*, *A. flavus*, *A. fumigatus*, and *Phytum* spp. Based on the results, it can be concluded that eight isolates of cellulolytic fungi have the potential to be used as agents producing cellulase enzymes which are capable of hydrolyzing cellulose in feed.

Keywords: Cellulolytic fungi; Detection; Evaluation; Identification; Pliek U

Introduction

High cellulose in a feed ingredient (forage) will limit the digestibility of livestock. Cellulase enzyme is known to have the ability to hydrolyze cellulose in feed. Cellulase is a commercial enzyme with the third most demand on the world market, which can degrade cellulose and is a synergy product in the cellulase enzyme group (Lokapirnasari *et al.* 2015). In addition, cellulase enzymes are very effectively used in various commercial sectors (various applications), including agriculture, animal science, fisheries and food science (Patel *et al.* 2019; Ejaz *et al.* 2021; Siva *et al.* 2022). One source of the cellulase enzyme is Pliek U, a typical food from Aceh (Indonesia) which comes from fermented coconut fruit. The manufacturing process takes place through several stages: peeling, taking coconut meat, washing, grating, curing (fermentation) and drying in the sun. After drying, it is squeezed to remove the oil, drying again, then putting it in a container. In addition, the fermentation process takes place spontaneously and the presence of fermentation stages allows the growth of various microbial groups. However, the level of hygiene during the process affects the diversity of microbes that grow in Pliek U, as well as when it is marketed. Handling before the manufacturing process, during manufacture and when it is marketed, it is suspected that there are beneficial or detrimental microbial groups. The existence of microorganisms in Pliek U will also affect its quality. The microorganisms contained vary depending on the process and handling management during manufacture. Several studies succeeded in isolating microbes from Pliek U. Before fermentation, the beginning of fermentation and the end of fermentation found three groups of bacteria and six types of fungal isolates, then managed to find twelve fungal isolates from several isolation stages (Rinaldi *et al.* 2016; Ejaz *et al.* 2019). Yunilas *et al.* (2019) also found twenty bacterial isolates; five were potential isolates in hydrolyzing cellulose.

Cellulolytic fungi are a group of fungi that can produce cellulase enzymes. Enzymes are specific catalysts in metabolic processes, playing a role in accelerating chemical reactions. In animal science/fisheries, cellulase

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enzymes can hydrolyze cellulose into simple sugars. In the pharmaceutical field, cellulase enzymes are often used to facilitate digestion or widely used to hydrolyze cellulose into its derivatives so that they can be used as additives for pharmaceutical products, namely methylcellulose, ethyl cellulose, hydroxypropyl methylcellulose, hydroxypropyl cellulose and carboxymethyl cellulose which are often used as coatings, binders, fillers, disintegrant and lubricant in the tablet manufacturing process, and is often used as a suspending agent (Adrio and Demain 2014; Earlia *et al.* 2019).

Microbes including fungi generally grow and develop according to the substrate content. Substrates containing high structural carbohydrates will stimulate the growth of cellulolytic microbial groups. Substrates containing high non-structural carbohydrates will stimulate the growth of amylolytic microbial groups. Furthermore, substrates with high-fat content will develop lipolytic microbial groups. Likewise, the substrate contains high protein, so the dominant group of proteolytic microbes develops. However, on the same substrate, various microbes, including bacteria, fungi and yeast, can grow with different hydrolytic abilities. As reported by Yunilas (2016), cellulolytic microbial groups grow on substrates based on plantation and palm oil industry waste, namely bacteria, fungi and yeast. In Pliek U, various bacteria are also found (Yunilas *et al.* 2019).

Microbes can produce multiple enzymes (endo-β-1,4-glucanase, xylanase, mannanase and lignin peroxidase) and synergize with each other in hydrolyzing cellulose (Yunilas *et al.* 2013). Cellulase enzyme activity (endo-β-1,4-glucanase) occurs when cellulose cells making up the substrate have a cellulose cell structure that is more easily degraded so that carbohydrates in the form of cellulose induce the formation of cellulase enzymes from microbes (Yunilas 2016). Cellulase enzyme activity is influenced by the type of substrate and substrate composition (Yunilas *et al.* 2013). The results of the explanation above prove that in Pliek U, there are various fungi. The diversity of fungi obtained is influenced by the handling before, during and after manufacturing. Qualitative detection, identification and evaluation of cellulase enzyme activity is the first step to finding potential isolates that produce cellulase enzymes. Judging from the broad benefits of cellulase enzymes in both livestock and pharmaceutical fields, the author was interested in detecting, identifying and evaluating the cellulase enzymes activity of cellulolytic fungi from Pliek U as cellulose hydrolysis agent in feed.

Materials and Methods

Materials

The material used is a source of fungal isolates from Pliek U. The selective medium for isolating fungi uses modified selective agar media (Yunilas 2016), namely: 0.2% $NaNO_3$; 0.05% KCl; 0.05% $MgSO_4.7H_2O$; 0.001% $FeSO_4.7H_2O$; 0.05% KH_2PO_4; 0.04% yeast extract; 2% agar and 1% (CMC, xylan, lignin and mannan), chlorophenicol (1 mg in 100 mL of water), pH adjusted to 4.5. Test the ability to degrade cellulose using standard agar media (PDA with adding 0.5 mg CMC). Physiological salt (NaCl), HCl, NaOH, filter paper, aluminium foil, gloves, masks, cotton, alcohol, Aquadest and spirits.

The tools used in this study include incubators and autoclaves. Microscope, Erlenmeyer, Petri disk, disk disc, test tube, tube rack, pipette, micropipette, stirrer, vortex, bunsen, glass object, cover glass, measuring cup, beaker glass, thermometer, pH meter, hot plate, digital scales, oven, calliper and digital camera.

Method

The research method used is the exploratory method. A source of isolates was Pliek U, which came from coconut meat undergoing fermentation. To obtain fungal isolates, isolation was carried out using the pour plate method on modified selective agar media (Yunilas 2016). Morphological observations were macroscopically from growing isolates, including color, size, shape, surface and colony margins. Identification of fungi was carried out based on colony morphology observations, including macroscopic and microscopic observations. The isolates obtained were purified using the streak plate method. The pure isolates were then tested for their ability to qualitatively degrade cellulose (enzyme activity) using the diffusion method. Potential isolates were selected based on the highest apparent zone ratio to the diameter of the colonies planted.

$$\text{Apparent zone ratio} = \frac{\text{Clear zone diameter} - \text{Colony diameter}}{\text{Colony diameter}}$$

Research procedure

Pliek U detection: Fungal detection was carried out using the isolated source from Pliek U. One gram of sample was taken and dissolved in 9 mL of sterile water containing 0.85% physiological NaCL, then serial dilutions were carried out up to 8–10. The results of the dilution series were taken at 1 mL and then inoculated into a petri dish that had been poured with selective agar media using the pour plate method. Media that has been inoculated and incubated for 24–48 h at room temperature to observe the growth of fungi. The growing colonies were observed for their morphology, including colour, size, shape, elevation and edges. Furthermore, the fungal colonies grown on the selective medium were transferred again to the new medium, and purification was carried out until a single colony was obtained. The pure isolate that has been obtained is stored in potato dextrose agar (PDA) slanting agar. Then the observation of the characterization of the fungi includes macroscopic and microscopic morphological

tests. Macroscopic observation of the fungus morphology was carried out during isolation. Isolates that managed to grow on selective media were observed and the colony's shape, color, size, edges and elevation were recorded.

Identify Pliek U: Observation of the morphology of the fungi (microscopically) was carried out by making a microculture (block square) of the isolates of the fungi as follows: Prepare PDA media in a Petri dish and let it solidify, then cut it into 1 × 1 cm pieces (block pieces of agar). Then one part of the agar block is taken and placed on a glass object in a petri dish covered with filter paper moistened with sterile distilled water. Then the test sample colonies were inoculated on all four sides of the agar block and covered with a cover slip. All work is done aseptically. Then incubate at 25–29°C for 5–7 days and after the incubation period, the preparations are observed under a microscope. The results of Pliek U identification will be compared with data in the Samson *et al.* (1981) reference book.

Evaluation of Pliek U: Enzyme activity testing to determine its ability to qualitatively degrade fibre (cellulose) in fungi according to each procedure. Cellulolytic microbes were indicated by forming a clear zone around the isolate. To see the clear zone so that it is more apparent, a qualitative test was carried out by pouring 5 mL of 1% Congo red reagent on the surface of the medium for 24 h. The wider the clear zone produced, the higher the enzyme activity produced in degrading cellulose. Furthermore, the fungi that produced the most comprehensive clear zone were purified for species identification.

Results

Detection of the fungi from Pliek U

Detection of cellulolytic fungi from Pliek U using the pour plate method. Pliek U is a typical Aceh food derived from fermented coconuts. The detected isolates could grow on modified selective media (Yunilas 2016) with the addition of Carboxy Methyl Cellulosa/CMC, xylan and tannic acid substrates. After 72 h of incubation, the morphology of the fungal colonies was observed. Then it is purified by cutting it a little to transfer it to PDA media. The results of the detection of cellulolytic fungi on a modified selective agar medium obtained nine fungi isolates. The fungal isolates obtained had various morphological characteristics ranging from colony color, colony size, colony shape and colony surface to colony margins, as shown in Table 1.

Identification of fungi originating from Pliek U

Identification results obtained nine isolates, of which two isolates came from the same strain (*Nigrospora* spp.) in isolates P1 and P4, has a classification Kingdom: Fungi; Phylum: Ascomycota; Class: Sordariomycetes; Ordo: Amphisphaeriales; Family: Apiosporaceae; Genus: *Nigrospora*; Species: *Nigrospora* spp. Six isolates came from various strains, namely isolates P3 (*Geotrichum* spp.), has a classification Kingdom: Myceteae; Phylum: Amastigomycota; Class: Deuteromycetes; Ordo: Moniliales; Family: Moniliaceae; Genus: *Geotrichum*; Species: *Geotrichum* spp. P5 (*Aspergillus niger*), has a classification Kingdom: Myceteae; Phylum: Mycota; Class: Deuteromycetes; Ordo: Moniliales; Family: Moniliaceae; Genus: *Aspergillus*; Species: *A. niger*. P6 (*Rhizoctonia solani*), has a classification Kingdom: Fungi; Phylum: Deuteromycota; Class: Deuteromycetes; Ordo: Agonomycetales; Family: Agnomycetaceae; Genus: *Rhizoctonia*; Species: *R. solani*. P7 (*A. flavus*), has a classification Kingdom: Fungi; Phylum: Ascomycota; Class: Eurotiomycetes; Ordo: Eurotiales; Family: Trichocomaceae; Genus: *Aspergillus*; Species: *A. flavus*. P8 (*A. fumigatus*), has a classification Kingdom: Fungi; Phylum: Ascomycota; Class: Eurotiomycetes; Ordo: Eurotiales; Family: Aspergillaceae; Genus: *Aspergillus*; Species: *A. fumigatus*. P9 (*Pythium* spp.) has a classification Kingdom: Fungi; Phylum: Mastigomycotina; Class: Oomycetes; Ordo: Peronosporales; Family: Phytiaceaea; Genus: *Phytium*; Species: *Pythium* spp. One isolate did not grow well so it could not be identified. Identification results are shown in Fig. 1.

Cellulolytic enzyme activity from Pliek U

The ability to degrade fiber can describe the activity of enzymes produced by a microbe. Fungal isolates with high cellulase enzyme activity can hydrolyze cellulose into glucose and are qualitatively indicated by forming a wide clear zone around the colony. The results of precise zone measurements for each isolate are presented in Table 2.

Discussion

Nine fungal isolates indicated the ability to produce several enzymes simultaneously (cellulase, xylanase, mannanase and ligninase) indicated by forming a clear zone around the isolates. The nine fungal isolates had various morphological characteristics: shape, size, elevation/surface, edges/edges and colony colour. The differences in the morphology of the fungal colonies indicated that the isolates were from different types of fungi. Some fungi grow faster than others, as indicated by the size of the bacterial colonies, which are larger than other colonies. Kowalski and Cramer (2020) stated that the morphology of the colonies produced would vary from a variety of sources.

The isolates P1 and P4 belong to the *Nigrospora* spp., which has conidia that are black, solitary in shape and unicellular which have phytopathogenic, endophytic and saprobic properties in different hosts (Hao *et al.* 2020). In addition, Rathod *et al.* (2014) stated that *Nigrospora* spp. capable of producing antimicrobial compounds that can control pathogenic fungi *Fusarium oxysporum*,

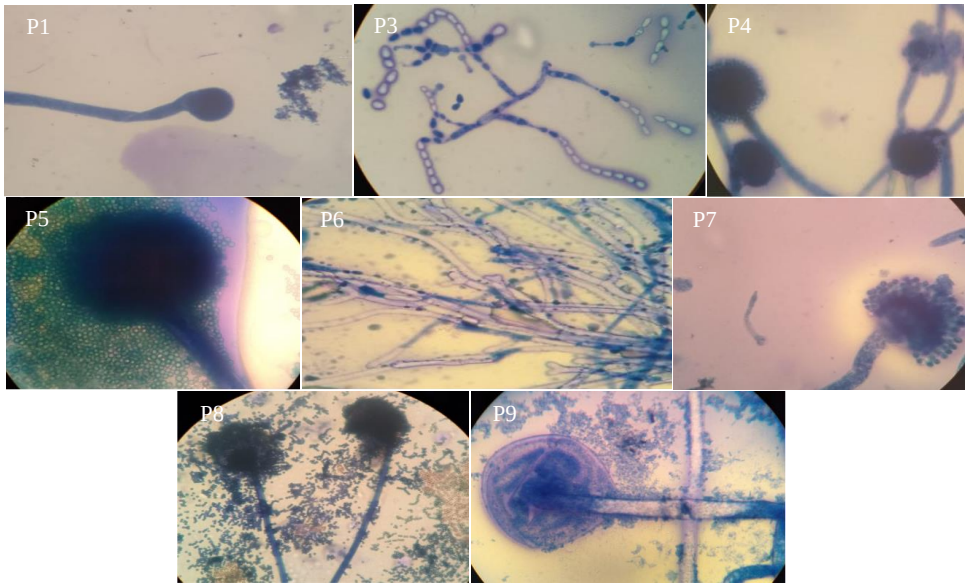

Fig. 1: Identification of fungi originating from Pliek U
P1 and **P4**: *Nigrospora* spp.; **P3**: *Geotrichum* spp.; **P5**: *Aspergillus niger*; **P6**: *Rhizoctonia solani*; **P7**: *Aspergillus flavus*; **P8**: *Aspergillus fumigatus*; and **P9**: *Pythium* spp.

Table 1: Morphological characteristics of fungi isolated from Pliek U

Isolate	Fungal colony morphology				
	Shape	Size	Elevation	Edge	Color
P1	Irregular	Medium	Raised	Filamentous	Fine white
P2	Circular	Medium	Flat	Curled	Greenish white
P3	Circular	Small	Umbonate	Filamentous	White
P4	Irregular	Small	Flat	Filamentous	Fine white
P5	Circular	Large	Flat	Curled	Dark brown
P6	Irregular	Large	Raised	Filamentous	White
P7	Irregular	Medium	Raised	Filamentous	Yellow
P8	Irregular	Medium	Raised	Undulate	White
P9	Irregular	Small	Umbonate	Undulate	White

Notes: P1 and P4 = *Nigrospora* spp., P3 = *Geotrichum* spp., P5 = *Aspergillus niger*, P6 = *Rhizoctonia solani*, P7 = *Aspergillus flavus*, P8 = *Aspergillus fumigatus* and P9 = *Pythium* spp.

Table 2: Diameter of clear zone and hydrolysis/cellulolytic index of fungi isolates from Pliek U

Isolate	Clear zone (mm)	Hydrolysis index/cellulolytic index
P1	26.0	2.25
P2	15.0	0.87
P3	22.5	1.50
P4	29.0	1.90
P5	26.5	1.94
P6	41.0	3.55
P7	43.5	4.11
P8	43.0	3.78
P9	30.0	2.00

Notes: P1 and P4 = *Nigrospora* spp., P3 = *Geotrichum* spp., P5 = *Aspergillus niger*, P6 = *Rhizoctonia solani*, P7 = *Aspergillus flavus*, P8 = *Aspergillus fumigatus* and P9 = *Pythium* spp.

Trichophyton mentagrophytes, *Microsporum canis*, *Streptococcus aureus* and *Escherichia coli* bacteria.

The isolate P3 belongs to the *Geotrichum* spp. which has macroscopic characteristics with white colony color, thin hyphae, smooth surface and round shape.

Meanwhile, if microscopically, it does not show the presence of long filaments, branching structures with septa which then break off to form arthroconidia (Kandi *et al.* 2020). In addition, Rinaldi *et al.* (2016) succeeded in obtaining six species of fungi that play a role in the process of making Pliek U, including *Geotrichum* spp. while Asril *et al.* (2019) succeeded in getting nine species of fungi that play a role in the process of making Pliek U, one of which is *Geotrichum* spp.

The Isolate P5 belongs to the *A. niger* strain, which has a conidia head shape, seriation, vesicle diameter, yellowish brown to black colonies and spherical spores (Nyongesa *et al.* 2015). Some of the fungi from Pliek U that were successfully obtained were *A. niger*, one of the most common species of the genus *Aspergillus* causing a disease called black mould on certain fruits and vegetables such as grapes, onions and peanuts. This fungus is ubiquitous in the soil and is commonly reported in indoor environments. *A.s niger* can cause aspergillosis in humans, particularly among horticultural workers who inhale peat dust, which can be rich in *Aspergillus* spores (Padmavathi 2015; Asril *et al.* 2019).

The isolate P6 belongs to the *R. solani* strain, which shows slightly melanized hyphae and sclerotia, which are irregular and brown. In addition, microscopically, it showed that the hyphae branched and the hyphae narrowed and formed septa at a short distance from the point of origin of the hyphal branches and the absence of clamp connections, conidia and rhizomorphs (Moni *et al.* 2016; Al-Fadhal *et al.* 2019; Senapati *et al.* 2022).

The isolate P7 belongs to the *A. flavus* strain macroscopically, known as velvet mould. The color of the colonies is yellow to green or brown with yellowish to red-

brown. While microscopic, the conid heads usually radiate and divide to form loose columns (Okayo *et al.* 2020). In addition, Fakruddin *et al.* (2015) reported that *A. flavus* has thick-walled, colourless conidiophores and elongated vesicles.

The isolate P8 belongs to the *A. fumigatus* strain, macroscopically, the colonies appear greenish and have insulating hyphae. In addition, microscopically, it is characterized by a chain of small oval conidia attached to the end of a continuously coiled row of sterigmata on the vesicle (Becchimanzi and Nicoletti 2022). The isolate P9 belongs to the strain *Pythium* spp. macroscopically. It looks like a white colony, while microscopically, it has a round sporangium (Chen *et al.* 2021).

From the screening, the index of cellulolytic activity (enzyme activity qualitatively) was more significant than one on agar media containing CMC, indicating that nine fungal isolates had the potential to degrade cellulose so that these fungi could be grouped into cellulolytic fungi. However, the area of the clear zone and the resulting cellulolytic index can illustrate a fungal isolate's ability to degrade fiber. The clear zone formed from fungal isolates from Pliek U ranged from 15.0–43.5, with a cellulolytic index ranging from 0.87–4.11. It can be seen that the clear zone and the cellulolytic index formed have various values. This illustrates that Pliek U can grow various fungi with different abilities from the same medium. The cellulolytic index of more than one includes the potential to degrade cellulose. Abe *et al.* (2015) reported that seven of the 19 moulds with cellulase activity were considered good producers with a cellulolytic index ≥ 2.0.

Cellulolytic fungi can hydrolyze natural materials containing cellulose into more straightforward products. The presence of cellulose in organic materials, including Pliek U, will induce the synthesis of cellulase enzymes so that the fungi will secrete cellulase enzymes. The ability to secrete cellulase enzymes of each fungus varies depending on the fungus gene, so high, medium and low cellulolytic index values will be produced. This will reflect how strong or weak the fungi are in degrading fibers such as cellulose.

The selection of fungal isolates was carried out by testing the ability of the fungi to degrade fiber (cellulose) in a medium containing CMC. Selection aims to obtain fungi isolates that utilize existing carbon sources (CMC). This can be seen from the clear zone around the fungal colonies. The clear zone formed indicates that the fungal isolate produces cellulase enzymes. Potential cellulolytic fungi isolates were indicated by the area of the clear zone formed and the high hydrolysis index obtained on agar media containing cellulose (CMC). Febriyanto *et al.* (2015) reported that RA2 isolate had a cellulolytic index 2.5. Naresh *et al.* (2019) reported that the KFX-40 isolate had the highest cellulolytic index of 3.42. Reddy *et al.* (2017) reported that JCEN 1 and RW isolates were potential isolates because they had a cellulolytic index of 2.5 (JCEN 1) and 2.0 (RW).

Conclusion

Fungi isolated from Pliek U have great potential in hydrolyzing cellulose in feed. The results of the detection of fungi growing on Pliek U obtained nine fungi isolates with the ability to hydrolyze cellulose ranging from 0.87–4.11. However, isolate P2 with a hydrolysis capacity of 0.87 did not grow well, making it challenging to identify. Differences in morphological characteristics and cellulolytic index characterize the various fungal isolates produced. Fungal isolates that have the potential to hydrolyze cellulose are those that have an enzyme activity value (cellulolytic index) > 1. Several strains of isolates have the potential as cellulose hydrolyzing agents and therefore play an important role in the livestock and pharmaceutical fields.

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Author Contributions

Y conceptualized and designed the study, elaborated the intellectual content, and performed the literature search, data acquisition, statistical analysis and manuscript preparation, NG and H carried out experimental studies performed the literature search, data acquisition, analyzed data and reviewed manuscript, LW and EY elaborated the intellectual content performed the literature search, reviewed manuscript and guarantor, MIAN and RA performed the literature search and data acquisition.

Conflict of Interest

All authors declare no conflict of interest.

Data Availability

Data presented in this study will be available on a fair request to the corresponding author.

Ethics Approval

Not applicable to this paper.

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