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Abstract. Hydrolysis of keratin waste by fungi is an alternative biotechnology for recycling and valorization by utilizing its keratinolytic activities. The purpose of this study was to isolate the keratinolytic fungi and to test the degradation ability of chicken feather keratin. Crocodile feces and soil samples were collected from crocodile breeding farm in Asam Kumbang, North Sumatera. Casein and keratin of basal feather agar of 1% was used to isolate keratinolytic fungi. Fungal isolate was grown in feather meal broth incubated at 28°C and shake at 180 rpm using shaking orbital. Remain chicken feather was weighted after application of keratinolytic fungi. After 4 days of incubation two fungi showed to have clear zone around their colony. THB7 was found to have relatively high hydrolysis zone in casein, while FB4 degraded more keratin in keratin agar. Most feather was degraded in 10, 12, and 16 days in THB7, FB4, and THB4 application respectively. THB4 showed to degrade feather to 1.6 g, while FB3 and FB4 remained feather to 2 and 3.4 of 10 g respectively. Further study includes molecular identification, characterization and keratinase production should be done.

1. Introduction

Million tons of feather and hair/bristle waste are produced every year by the food processing industry from poultry and slaughterhouse [1]. Unfortunately, the environment cannot properly process these kind of wastes which leads to the disruption of the surrounding ecosystem especially the soil biota ecosystem. Therefore, keratinase involvement is necessary to convert the abundant keratin waste in nature considering that it does not require high economic costs, is rich in nutrients and can be considered as an eco-friendly approach [2]. Currently, the microorganism is the most common contributor to keratinase production for application purposes.

Keratinolytic microorganism is currently widely applied in the field of biotechnology such as in detergent formulation, leather industry, pharmacy, and cosmetics. In addition, keratinase can be used for bioconversion of animal feed and waste agro-industries into specific useful products such as amino acid (serine, cysteine, and proline), peptides, and biofertilizers [3].

In addition to keratinase-producing bacteria such as *Bacillus*, *Chyseobacterium*, *Stenotrophomonas*, and *Fervidobacterium* [4,5,6,7], fungi has also been explored for potential application in environmental biotechnology. Fungi can grow on a low-cost substrate and produce enzyme into large amounts in culture [8,9]. Keratinolytic fungi produce keratinase to specifically degrade keratin, which

is the main component of keratin substrates in cornified epidermis, hair, feathers, nails, horns, hooves, scales, and wool [10]. Keratin is composed up to 90% of protein, especially in feathers [11].

Keratinolytic microorganism is commonly found from different habitat and sources. However, its exploration is limited to some sources only, such as epidermis, soil, poultry farm waste, and sea sponge [12,13,14,15,16]. Such source supports substrate availability to meet the carbon and nitrogen sources for growth of microorganisms.

The purpose of this study was to isolate keratinolytic fungi from a different source as in previous study. In this study, fecal and soil samples of crocodile breeding farm were used as source of fungal isolates. The crocodile is feed with a whole duck, chicken, and bird, and sometime wild crane in crocodile pond. Natural accumulation of their feathers in feces and soil in the farm were create suitable environment for keratinolytic microorganisms including keratinolytic fungi. The fungi may use the feather as its C and N source. Degradation ability of feather was measured as weight of remain feather after application with keratinolytic fungi.

2. Materials and methods

2.1. Isolation of Keratinolytic Fungi

Crocodile feces and soil samples were collected from crocodile breeding farm of Asam Kumbang Medan Selayang, Medan, North Sumatra, Indonesia (98°37'11.24"E 3°33'58.38"N). The samples were placed in sterile polyethylene bags, immediately transported to laboratory, and kept at 4°C. A 10 g sample of soil and dry crocodile feces was diluted in 90mL of sterile aquadest and shaken at 120 rpm for 10 minutes. Mixture was serial-diluted to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}. Dilution of 10^{-7}, 10^{-8}, 10^{-9} was poured and spread on modified feather agar of Saber *et al*. [17] containing 0.5 g $MgSO_4.7H_2O$, 0.1 g KH_2PO_4, 0.01 g $FeCl_2$, 0.005 $ZnSO_4.7H_2O$, and 15 g agar in 1000 ml sterile aquadest. pH was adjusted to 7.8. Media was added with 2% broiler chicken feather as sole C and N source. Culture was incubated for 3-4 days at 37°C. Growing fungi were used for further study.

2.2. Screening of Keratinolytic Fungi on Casein Agar

Keratinolytic fungi of previously isolated was sub-cultured in modified 1% casein agar (VWR Chemicals, E666-500G) [17] and incubated at 37°C for 4 days. Coomassie Blue solution (CBB R-250 2.5 g l^{-1} in CH_3OH + glacial CH_3COOH +aquadest in 50:10:40 ratio) [18] was added to the active fungal growth. Observation of clear zones around fungal colony was conducted to select proteolytic fungi.

2.3. Reconfirmation of Keratinolytic Fungi on Keratin Feather Agar

Fungal isolate was reconfirmed for its keratinolytic ability by growing it in modified of keratin agar [17] using 1% whole feather. Culture was incubated at 37°C for 4 days. Coomassie Blue solution was added as previously done. Keratinolytic activity was observed as a clear zone around fungal colony.

2.4. Whole and Powder Feather Degradation in Broth Medium

Spore of fungal isolates of 10^7-10^9 spore/cm^{-3} were cultured in 250 mL of feather meal broth containing 1% keratin feather and incubated at 37°C for 96 h [17, 20]. A 3 mL of 2% Tween 20 sterile was added in culture media to reduce surface tension. Similar work was conducted for whole feather but used 10 g of chicken feather rather than feather powder. Media feather meal broth without fungal spore inoculation was used as control.

2.5. Preparation of Chicken Feather

Preparation of chicken feather was performed with modified method of Călin *et al*. [19]. Chicken feather from a local farm was surface sterilized with ethanol and subjected to be washed in running water for 10 minutes. The feather was dried for 2 hours under sunlight and dried in oven at 60°C for 1 hour. Chicken feathers were cut into small piece of 2 cm length and autoclaved at 121°C for 30 min.

Feather powder was prepared by mashing feather using *mortar and pestle*. Feather powder was stored at 8°C for future use.

2.6. Feather Weight Measurement
Degradation of chicken feather was periodically observed every 2 days for 20 days by weighting remain chicken feather after fungal application.

3. Results

3.1. Isolation of keratinolytic fungi
Three fungal isolates were successfully obtained from fecal and soil sample of crocodile breeding farm. FB4 was isolated from crocodile's feces and two THB4 and THB7 were isolated from soil sample. The fungal isolates were seen in feather agar after 4 days of incubation.

3.2. Screening of proteolytic and keratinolytic fungi
Hydrolytic zone was clearly seen in casein compared to that of keratin agar (Fig. 1). It seemed that time needed for hydrolysis of casein and keratin were similar, in 4 days. However, each fungal isolates showed to have different ability in hydrolysing protein. Three fungi isolates had proteolytic and keratinolytic activities as it could be seen as clear zone around fungal mycelium growth. FB4 showed to grow fast with wide area of mycelium in keratin agar (Fig. 1f) followed by THB7 and THB4. This indicated that all isolates utilized keratin as C and N source.

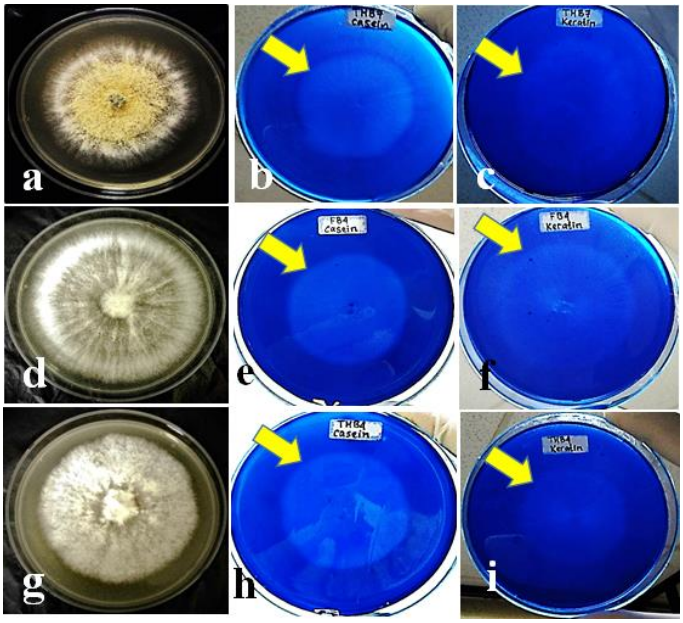

Figure 1. THB7 colony on agar plate (a) with 1% casein (b) and (c) 1% feather powder; FB4 colony on agar plate (d) with 1% casein (e) and (f) 1% feather powder; and THB4 colony on agar plate (g) with 1% casein (h), and (i) 1% feather powder after four days of incubation at 37°C. Each isolates showed a clear zone (yellowed arrow) which indicates hydrolytic ability of three fungal isolates.

The fungal growth was observed by using casein and keratin media from 24 h to 96 h. Both casein and keratin media showed hydrolysis zones at 96th hour. In casein medium, THB7 (39.3 mm) was the largest zone and THB4 (37.5 mm) and FB4 (36 mm) were the second largest. On the other hand, the largest hydrolysis zone in keratin medium was FB4 (48.6 mm), followed by THB7 (45.2 mm) and THB4 (29.7 mm). The utilization of chicken feather as the source of C and N by keratinolytic fungi was to grow hyphae. Chicken feather can maintain the existence of fungi that grow into several groups

of hyphae to form mycelium. Therefore, FB4, THB4 and THB7 isolates are highly recommended to be grown in growth media containing chicken feathers. Not only useful for fungal growth, chicken feathers as the only source of carbon and nitrogen will make the fungi capable of producing keratinase enzyme which can be used for application purposes.

Chicken feather is known to contain alpha and beta keratin which are resistant to degradation. The alternative solution is to use keratinase from fungi to determine the alpha and beta keratin bonds of chicken feather as a whole through combining different keratinolytic fungi. Friedrich et. al. [21] reported that about 54% of fungal growth through keratin degradation was caused by enzyme secretion. It has been explained that microbial keratinase could be used to increase the nutritional value of animal feed [22]. In this case, keratinolytic enzyme has considerable benefits in the field of biotechnology involving keratin waste from poultry industries.

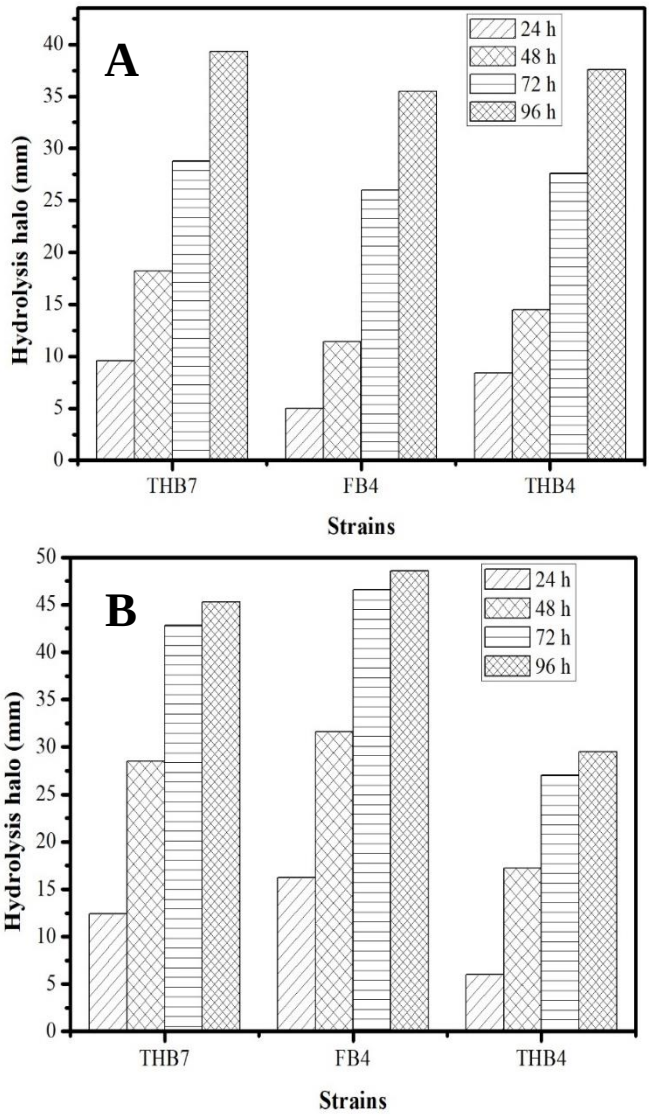

Figure 2. Diameters of hydrolysis zone of different keratinolytic fungal isolates on (a) casein and (b) keratin agar plate.

3.3. Degradation of chicken feathers in liquid media

Keratinolytic fungal spore were suspended into feather broth media added with a whole chicken feather as C and N source. It was observed that THB7 degraded chicken feather faster in 10 days of incubation (Figure 3), followed by FB4 and THB4 in 12 and 16 days of incubation. Chicken feathers weight differ after fungal application in which in FB4 feather remain was 1.6 g, followed by THB7 and THB4 with 2 g and 3.4 g respectively compared to that of control.

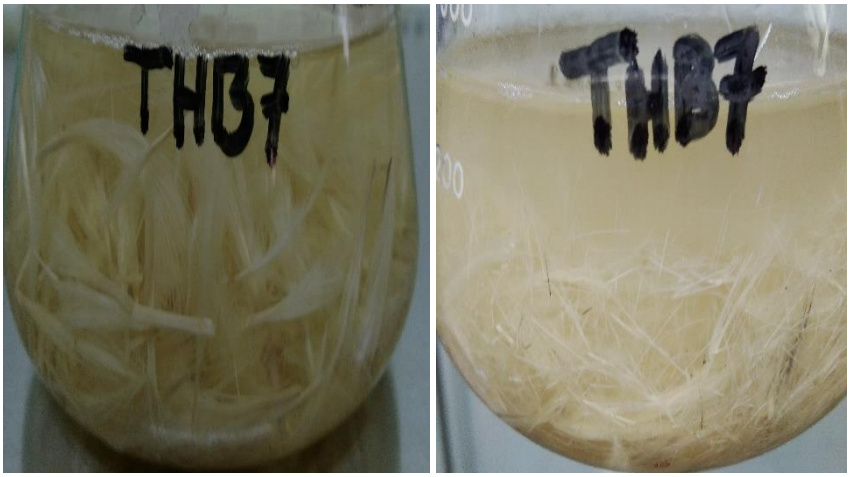

Figure 3. Chicken feather degradation by THB7 strain (10 days of fermentation)

4. Discussion

Keratinolytic fungi is commonly found in poultry farms due to its huge amount of keratin of feather waste [23]. Our showed that keratinolytic fungi could also be found from crocodile feces and soil in crocodile breeding habitat. This might due to whole duck and its relatives as crocodile feed. Ninety percent of chicken and bird wings contain β keratin and fibrous protein [24,25] which is difficult to degrade by the digestive system and return to the environment. To our knowledge, fungi with keratinolytic activities are expected to grow well in crocodile breeding habitat with plenty of feather waste.

Three keratinolitytic fungi isolates FB4, THB4 and THB7 were isolated on selective media of feather agar. Each fungal isolate was evaluated for its keratinolytic activity using casein and keratin enriched medium. FB4 found in crocodile feces was successfully degrades chicken feather. This is corresponding with its ability to produce larger clear zone in feather agar despite its relatively lower proteolytic activity in casein agar compared to that of THB7. Unlike other proteases, production of extracellular keratinase might induced in media containing keratin [26].

It seemed that after 96 hours of incubation substrate hydrolysis was shown by each fungi indicating by its clear zone after adding with Coomassie Brilliant Blue solution. Fungal isolates with larger hydrolytic zone was THB7 with 39.3 mm in agar casein, followed by FB4 (48.6 mm) in the keratin agar. Similar result were observed in keratinolytic fungi of *Aspergillus* genus [26] which degraded keratin 96 hours after incubation. FB4 showed to reduce whole feather weight from 10 g to 1.6 g in 12 days. However in 10 days THB7 degraded whole feather to 2 g in 10 days.

Many keratinolytic activity of fungi for example *A. niger*, *P. marquandii*, and *Doratomyces* were reported to show their proteolytic activity in gelatin, keratin, wool, and horn layers [26,27]. Wihout doubt these fungi play an important role in the ecological system to recycle carbon (C), nitrogen (N) and sulfur in keratin [28], therefore, keratinolytic fungi isolates are generally obtained in waste of animal hair, zoological garden, poultry farms [29,30,31]. It was interesting that this kind of fungi was also found in marine sponges [32]. So far, no report of keratinolytic fungal was from crocodile

breeding farm. Our results showed that our keratinolytic fungal isolates might be potential as keratinase producer or as keratin degrader. Further study should be taken.

5. Conclusion

Our study found three keratinolytic funga isolated from crocodile breeding farm of Asam Kumbang, Medan Selayang. FB4 was isolated from the crocodile's feces and THB4 and THB7 were isolated from soil sample. THB7 showed to hydrolyse more casein with clear zone of 39.9 mm in casein agar, while FB4 degrade more keratin with clear zone of 48.66 mm in keratin agar. FB4 required 12 days to reduce whole feather form 10 to 1.6 g. Further study in molecular identification, characterization and keratinase production of these three fungal isolated should be done.

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