Screening of Keratinolytic Fungi for Biodegradation Agent of Keratin from Chicken Feather Waste

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Abstract. Chicken feathers waste are an abundant source of keratin protein. However, keratin contained in chicken feather waste is difficult to degrade so that its utilization becomes limited. Environmentally friendly degradation efforts require keratin proteases in addition to common proteases. Therefore, microorganisms that effectively degrade keratin are needed to recycle the waste. This study aims to isolate and screen fungi that was capable of degrading chicken feather keratin. Isolate with the highest degradation activity index for hydrolyzing chicken feather flour and common protein substrate was selected as an agent for chicken feather keratin biodegradation. The identification was carried out phenotypically and molecularly with phylogenetic analysis of the region gene sequences. The results of the study found \textit{Talaromyces sayulitensis} GF11 as a candidate for chicken feather keratin biodegradation agent. It is a new strain of keratinolytic fungi which was first published.

Keywords: Keratinolytic fungi, chicken feather waste, biodegradation agent

1. Introduction
The poultry processing industry produces many by-products in the form of chicken feather waste [27]. Chicken feathers contain 90% protein as keratin [12]. Keratin is difficult to degrade become soluble compound [13]. The continuous production of chicken feather waste causes chicken feather waste to become a source of pollution worldwide [18].

Microbes are known as sources of enzymes. Microbes produce enzymes for their life and this is used in various human needs. Keratinolytic fungi from chicken feather waste produce keratinase and protease enzymes to hydrolyze keratin into dissolved proteins and free amino acids [28]. Microbial growth in chicken feather waste can be developed as a keratin degrading agent in chicken feather waste [3].

Keratinolytic fungus can support management of chicken feather waste and the application of biotechnology in various fields of human activity that are environmentally friendly. Keratinolytic fungi can also be developed in many industries that use basic ingredients containing keratin [29] and the production of biological fertilizers [11]. Keratinolytic fungi
that have been widely studied are from Ascomycetes family members. One species of fungus that actively degrades keratin is Onygena corvina [10].

The presence of indigenous fungi in chicken feather waste has the potential as a keratin degrading agent in chicken feathers. In this connection, it is necessary to obtain the isolate. The purpose of this study is the isolation and selection of fungal strains that have the highest activity in degrading chicken feather waste. Fungal strains that have the highest keratinolytic activity were identified phenotypically and molecularly.

2. Materials and Methods

2.1 Isolation of Keratinolytic Fungi

Soil mixed with degraded chicken feather waste around the broiler production house in Jember, Indonesia was chosen as a source of fungal isolates. Sample was obtained using a sterile scalpel and the sample was placed in a sterile poly propylene bag. The physical and chemical conditions of the environment at sampling location was observed to obtain environmental data where the isolates grew.

In this study chicken feathers were processed into flour as a constituent material for isolation and test for keratinolytic fungal degradation activity. Chicken feathers from broiler production houses was washed with water, dried, and rinsed with distilled water. The feathers were incubated at 45°C for 2 days until dry and ready to be mashed into chicken feather flour with size of less than 200 µm.

The medium used for fungal isolation were broth and solid media of Mineral Chicken Feathers (MCF). The composition of liquid medium was 0.5% glycerol, 0.05% K$_2$HPO$_4$, 0.1% yeast extract, and 1% chicken feather flour. The MCF solid medium was made by adding 1.5% agar without glycerol to MCF liquid medium. The liquid media used for the beginning of microbial enrichment and was continued with the isolation of fungi which were able to degrade chicken feather flour. In the agar media, streptomycin 0.1% was added to inhibit bacterial growth [31]. Media of agar MCF was also used for culture maintenance. This study used Skim Milk agar medium to assay the activity of protein hydrolysis. The composition of the medium were consists of 10% skim milk and 1.5% agar [5].

Isolation was initiated by preparing a suspension of the composite sample of 25 grams in 250 ml of physiological saline solution (0.85% NaCl) (w/v). The homogenized sample suspension was transferred to MCF liquid medium as much as 10% of the medium volume. The inoculated medium was incubated at the orbital incubator (Stuart) at a speed of 180 rpm for 7 days at 30 oC. The culture was serially diluted and inoculated onto MCF agar medium. The culture was then incubated in an incubator at 30 oC for 7 days. Each colony that grows with different morphology and color was transferred onto MCF isolation medium without the addition of glycerol and yeast extract. Keratinolytic fungi that grow and produce clear zones around the colonies were isolated. The clear zone was observed after the plate area was colored with Coomasie Briliant Blue G250 [9].

2.2 Screening for Keratinolytic and Proteolytic Fungi

The ability of fungi isolates to degrade keratin chicken feathers was tested in a semi-quantitative manner by growing pure culture on MCF agar plates without the addition of glycerol and yeast extract. Culture plates were incubated at 30 oC for 4 days. The keratinolytic activity of each isolate was observed by calculating the clear zone index measured by the ratio of colony diameter to the diameter of the clear zone [26]. Each fungi isolate was tested with replications for three times. The difference in the ability of isolates to degrade keratin chicken feathers was analyzed by the diversity analysis test (ANOVA) $p < 0.5$ using the SPSS 15.0 program. Isolates capable of degrading keratin chicken feathers were also tested for their ability to hydrolyze skim milk protein.
2.3 Identification of Keratinolytic Fungi

2.3.1 DNA extraction, sequencing, and phylogeny analysis

Molecular identification was carried out on an isolate which have the highest degradation activity against keratin feathers. Firstly, production of fungi biomass was prepared by growing isolate for 7 days in the liquid media of yeast extract (YDB). The DNA was extracted from biomass of the fungal isolates using a DNA extraction mini kit i-genomic soil reagent (Intron Biotechnology, Inc.) according to the process indicated by the manufacturer.

The ITS region fragment in total DNA was amplified with the universal ITS primer using a PCR machine (Eppendorf, personal Mastercycler). Specific oligonucleotide primers used were ITS4 primer (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 primer(5'-GAAAGTAAAAGTCGTAACAAGG-3'). These primers were used to amplify regions of ITS1, 5.8S, and ITS2 region between 18S and 28S regions [22]. The DNA amplification conditions were 30 cycles consisting of predenaturation at 96°C for 7 minutes, denaturation 95°C 30 seconds, annealing at 52 °C for 30 seconds, elongation at 72 °C for 1 minute, and post elongation 72 °C for 7 minutes. The amplified PCR products of the appropriate size (fragments of about 700 base pairs) were confirmed by gel electrophoresis. Amplikon was checked in gel electrophoresis with a composition of 0.8% agarose gel in 1x TBE buffer traced at 100 V for 30 min, and stained with Ethidium bromide. Amplicon in the gel was observed in UV transilluminator and documented. PCR products were purified and sequenced at the 1st BASE company, Malaysia using the Applied Biosystems sequencer with the BigDye® Terminator v3.1 chemistry cycle sequencing kit.

Nucleotide sequences of fungi isolates were developed to produce contig sequence with the BioEdit Program for Windows. Contig sequence was analyzed homologically with other fungal nucleotide sequences found in the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov) using the BLAST program found on the website (http://www.ncbi.nlm.nih.gov/blast). The next sequence comparison was performed on strains that are closely related obtained in the other http://www.straininfo.net / strains / search and GeneBank sites. Operational taxonomic units (OTUs) use a 97% similarity limit as the same species [23].

Sequential phylogenetic analysis of ITS region was carried out with the molecular evolutionary genetic analysis (MEGA version 7.0) software [15] and used the statistical method 4 software package the Neighbor-Joining [24]. Phylogenetic tree construction used only sequences of fungi strains that have been published validly. The confidence level of taxonomic relationships in clusters was tested by bootstrap (1000 repetitions) [8].

2.3.2 Macroscopic and Microscopic Morphological Characterization

Macroscopic characters were studied by growing cultures in different media and growth conditions. Culture was inoculated on Potato Dextrose Agar (PDA) and TABM agar plates, using 90 mm petri dishes. The inculum was made from a spore suspension in a semi-solid agar solution containing 0.2% agar and 0.05% Tween 80 [19]. Spore inoculation using a micropipette by dropping in one point (0.5-1 μl per spot). Culture plates were incubated for 7 days 25 °C in darkness. Degree of sporulation, colony color and production of dissolved pigments are recorded. The colony color code used in the description refers to Kornerup & Wanscher (1967) [14]. For ascoma production, slide cultures with PDA media were incubated for up to four weeks. Macromorphological characteristics such as colony diameter, sporulation degree, sclerotia production, mycelia color and the presence of exudates, were then described. The microscopic character of fungi was observed in culture plates which were incubated for 1 to 2 weeks on PDA media. The characters observed were ascomata, asci, and ascospores. Character was documented using a stereo microscope. This microscope is equipped with Opti Lab devices.
3. Results and Discussion

3.1 Keratinolytic and Proteolytic Fungi

Nine fungal isolates from the soil sample could grow on medium containing chicken feather flour as the only source of nitrogen and carbon. All isolates grew and formed a clear zone significantly around the colonies (Figure 1a). The clear zone index indicates the ability of each isolate to degrade against keratin chicken feathers. Statistical analysis of the clear zone index between keratinolytic fungal isolates showed that GF11 was the best keratinolytic strain (Figure 1b).

![Degradation activity shown as clear zone (arrow)](image1)

![Comparison of degradation activity among fungi keratinolytic](image2)

Figure 1. Semi Quantitative Degradation Activity of Fungi Isolates Assayed on CFM Agar Medium Containing Chicken Feathers Keratin.

In this study, all isolates grew and were also able to hydrolyze skim milk with varying clear zone indices. Statistical analysis of the protein hydrolysis activity showed that GF11 isolates were able to grow in medium with simple protein sources with lower activity compared to other isolates (Figure 2). GF11 isolate has greater degradation activity against keratin chicken feathers as a complex protein than its activity against skim milk protein as a simple protein.

![Simple Protein Hydrolysis by GF11 Fungal Isolate and Other Strains in Skim Milk Agar](image3)

Figure 2. Simple Protein Hydrolysis by GF11 Fungal Isolate and Other Strains in Skim Milk Agar
Both of keratin proteases and simple protein protease degraded keratin to produce soluble proteins and free amino acids [16]. Keratin hydrolysis into a simple protein requires keratin proteases to convert complex structures into simple proteins. The protein can then be hydrolyzed by common protein proteases to be more soluble peptides and free amino acids

### 3.2 Identification of Keratinolytic Fungi

#### 3.2.1 Phylogenetic Analysis

Amplification of ITS region from the genome of the keratinolytic fungal isolate GF11 (Figure 3a) produced a DNA fragment of about 700 bp (Figure 3b). The ITS fragment after being sequenced and developed with the Bio Edit program produced fragments with sequences measuring 561 bp (Figure 4). The results of the alignment of the nucleotide sequences of these isolates with the reference function sequences in the NCBI BankGen showed homology of 99% with sequences of various species of the genus *Talaromyces* sp.

![Electrophoresis of genomic DNA and ITS Amplification Product of GF11 Isolate.](image)

Evolutionary distance analysis with the MEGA version 7 program [25] on ITS rDNA sequences in GF11 isolates compared to some types of Talaromyces species found that the isolates had the highest similarity with *Talaromyces sayulitensis* DTO 245H3 (99.79%) (Table 1).
Table 1. Genetic Distance Differences in Sequences of ITS Fragments between GF11 Strains and Various *Talaromyces* Reference Species Analyzed by MEGA7 Program.

| No | Species references          | Divergences | Similarities (%) |
|----|----------------------------|-------------|------------------|
| 1  | *Talaromyces aculeatus* CBS 289.48\(^T\) | 0,010468    | 98,95            |
| 2  | *Talaromyces allahabadensis* CBS 453.93\(^T\) | 0,172181    | 82,78            |
| 3  | *Talaromyces barcinensis* CBS 649.95\(^T\)   | 0,053864    | 94,61            |
| 4  | *Talaromyces coalescens* CBS 103.83\(^T\)    | 0,074242    | 92,58            |
| 5  | *Talaromyces dendriticus* CBS 660.80         | 0,062853    | 93,71            |
| 6  | *Talaromyces erythromellis* CBS 644.80\(^T\) | 0,095190    | 90,48            |
| 7  | *Talaromyces flavovirens* CBS 270.35\(^T\)  | 0,083480    | 91,65            |
| 8  | *Talaromyces sayulitensis* DTO 245H3        | 0,002082    | 99,79            |

The phylogenetic tree of ITS sequence showed that GF11 isolates were in one clade with DTO 245H3 *Talaromyces sayulitensis* [30]. The fungi that were outgroup in the phylogenetic tree were *Talaromyces allahabadensis* CBC 453.93\(^T\) [22]. Based on the phylogenetic analysis, GF11 isolates were identified as *Talaromyces sayulitensis* GF11 strain (Figure 4).

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![Figure 4. Phylogenetic trees that show the relationship of taxa between GF11 sample strains and various *Talaromyces* species constructed by the Neighbor-Joining method [21].](image)

Phylogenetic analysis of ITS region placed sequences of isolates of GF11 with high similarity with sequences of various *Talaromyces* species. The microscopic and macroscopic morphological characteristics of the fungus also showed characteristics similar to the *Talaromyces* genus in the *Talaromyces* section. Based on the taxonomic analysis of polyphasics, GF11 isolates were identified as *Talaromyces sayulitensis* GF11. The fungus was a new strain that has the closest kinship relationship with *Talaromyces sayulitensis* DTO 245H3, the origin of fungi from Mexico [30].
### 3.2.2 Characterization of Macroscopic and Microscopic Morphology

Description of the growth of *Talaromyces sayulitensis* GF11 which includes the characteristics of macromorphology and micromorphology shown in Figure 5. Growth on PDA media, the fungus has the color of a light brown colony with the same zoning and reverse color colony. Whereas on CFM media, the fungus has the same gray colony and the same reverse color colony. The microscopic morphology of the fungus on TABM media has hyphae diameter between 1-2 µm, forming ascospores, conidiophore branching of solitary phialides, and shapes of conidia is ellipsoidal (Figure 5).

![GF11 Fungi Isolate. Colony: Upper Left to Right Lane, Color Viewed from the Upper PDA Media Culture, Under PDA Culture, Top TABM Media Culture, Left to Right Bottom Lane, Hypha, Ascospore, Conidiophores and Conidia.](image)

Based on the description of *Talaromyces* published by Visagie et al. (2014) [30] showed that *Talaromyces sayulitensis* GF11 has these characteristics. The unique character of the strain of the fungus is the color of light brown colony and the type of conidiophoric branching in the form of solitary phialides.

At present, the *Talaromyces* genus is known as an important microbe for biotechnological purposes [32], biofertilizer production [6], enzyme production for deconstruction of lignocellulosic biomass [20] and producers of a series of anticancer compounds [32]. This study found that *Talaromyces sayulitensis* GF11 is one of the keratinolytic fungi present in the world [1, 2, 4, 7, 11, 17] and were first published as strains of the species *Talaromyces sayulitensis* which were able to degrade keratin chicken feathers.

### 4. Conclusion

In conclusion, among the nine keratinolytic fungi isolated, *Talaromyces sayulitensis* GF11 from Jember was a new strain of *Talaromyces sayulitensis* fungi. It is a potential agent for biodegradation of chicken feathers keratin.

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