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Isolation and Identification of Swan (*Cygnus olor*) Digestive Tract Cellulothic Bacteria to Support Fiber Degradation

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Abstract: Cellulolytic bacteria are bacteria that produce cellulase enzymes capable of degrading cellulose substrates. This study aimed to isolate and identify as well as measure the cellulase enzyme activity of cellulolytic bacteria isolated from the digestive tract of swans (*Cygnus olor*) in Mataram. The bacteria were isolated using de Man Rogosa Sharpe (MRS) selective media with the spread plate method. Then, the extracellular enzyme activity test was conducted by growing selected pure isolates on 1% Carboxy Methyl Cellulose (CMC 1%) media, followed by pouring 0.1% congo red solution and rinsing with 1M NaCl solution to determine cellulolytic activity (cellulolytic potential indicated by the appearance of a clear zone around the colony) and measuring the cellulolytic index. The bacterial isolation results obtained 4 isolates with the potential to support fiber degradation in animal feed. The highest cellulolytic index was produced by the isolate coded S_1, reaching 34.5 mm, while the lowest cellulolytic index was produced by 2 isolates coded J_{21} and I_2, reaching 18.8 mm.

Keywords: Cellulase enzyme; Cellulolytic; Fiber degradation; Swan

Introduction

The livestock industry is one of the strategic efforts to meet food needs in Indonesia. Especially the poultry livestock industry, which experienced rapid growth and became the spearhead in meeting national meat consumption needs (Ditjen PKH, 2017). This was reinforced by Dimyati (2018), who explained that the level of chicken meat consumption in 2017 reached 12.5 kg/capita/year with an increase of nearly 11% each year. The increase in meat consumption correlated with increased livestock productivity and feed efficiency.

Feed is one of the main keys to the success of a livestock business (Reski et al., 2021). Providing quality feed can affect the growth and survival of cultivated livestock. The high price of commercial feed rich in protein has become the main obstacle in developing livestock businesses, leading many farmers to use local feed as a substitute for commercial feed.

The utilization of agricultural waste materials as livestock feed was an alternative solution. Additionally, it helped reduce production costs. Agricultural waste materials include corn bran, rice bran, coffee husks, corn cobs, and other agricultural residues. However, these materials typically had low protein content and high crude fiber content. Crude fiber consists of cellulose, hemicellulose, and lignin, which are largely indigestible by poultry and act as fillers or bulky materials (Raharjo & Isnawati, 2022; Purnamasari et al., 2020; Wardah & Panjaitan, 2019; Wahju, 2004).

High cellulose content in feed materials can be hydrolyzed using cellulase enzymes because these enzymes have the ability to hydrolyze β-1,4-glycosidic bonds in cellulose molecules, thus producing glucose (Pulungan & Tumangger, 2018; Seprianto, 2017; Saratale

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et al., 2012). Cellulase enzymes can be produced by cellulolytic microbes, including fungi and bacteria. Several bacterial genera known for their cellulolytic ability include Achromobacter, Angiococcus, Bacillus, Cellulomonas, Cytophaga, Clostridium, Cellivibrio, Flavobacterium, Pseudomonas, Poliangium, Sorangium, Sporocytophaga, Vibrio, and Cellfalcicula (Rao, 1994), as well as Citrobacter, Serratia, Klebsiella, Enterobacter, and Aeromonas (Anand et al., 2010).

High fiber content in local feed could be degraded using appropriate technology, one of which involved utilizing bacteria capable of producing cellulase enzymes. These bacteria were found through isolation techniques in their habitats. Therefore, this study isolated and identified bacteria from the digestive tract of swans (*Cygnus olor*) with the aim of discovering bacteria that had the ability to produce cellulase enzymes to support the degradation of crude fiber in animal feed.

The high cost of commercial livestock feed, which was difficult for farmers to afford, has been a hindrance to increasing livestock productivity. Additionally, the high crude fiber content in local feed, especially from agricultural waste, made it difficult for poultry to digest, limiting the optimal utilization of feed derived from waste materials to reduce feed costs and improve livestock productivity, which correlated with production efficiency. Therefore, an alternative solution was needed, such as utilizing cellulase enzyme-producing bacteria to degrade the high crude fiber content in feed. This approach could enhance digestibility in poultry, thereby increasing production while reducing feed costs.

The aim of this study was to isolate and identify cellulolytic bacteria capable of producing cellulase enzymes to support fiber degradation in animal feed.

Method

Research Time and Location

The study was conducted from April to August 2023 at the Microbiology and Biotechnology Laboratory, Faculty of Animal Husbandry, Universitas Mataram.

Research Design

Bacteria isolation from the digestive tract of swans (*Cygnus olor*) was conducted on swans aged over 1 year and 2 months in the proventriculus, duodenum, jejunum, ileum, caecum, and colon. After isolation, bacterial isolates were purified to obtain pure isolates (single colonies), followed by bacterial identification. Bacterial identification involved macroscopic and microscopic observations, biochemical and physiological tests, as well as enzyme activity assays.

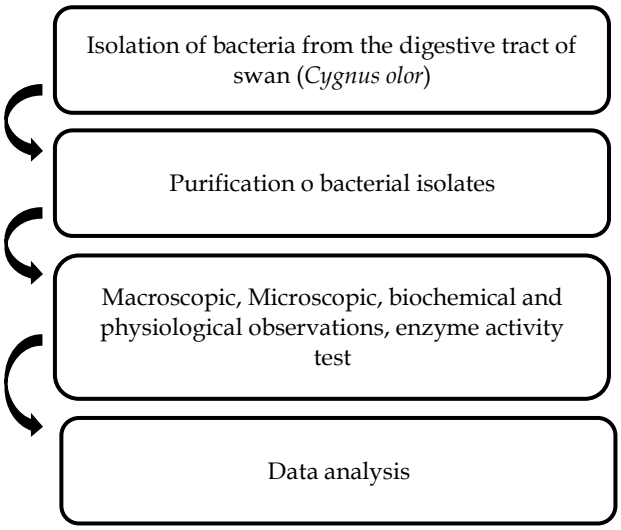

Figure 1. Research Design

Sample Preparation Swan Digestive Tract (Cygnus olor)

The digestive tract of approximately 8-month-old swans was obtained by taking sections including the proventriculus, jejunum, ileum, duodenum, caecum, and colon. Each section was cut into pieces (1-2 cm), then dissolved in sterile PBS solution and further diluted using serial dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}.

Sample Isolation and Purification of Bacterial Isolates

Sample isolates from dilutions were isolated on solid MRS media using the spread plate technique and incubated for 24 hours at 37°C. Bacteria that grew and exhibited different colony morphologies were then purified using the streak plate method and subsequently cultured in liquid MRS broth media.

Colony Morphology Characterization

Bacterial morphology characterization consisted of colony morphology (macroscopic) and cell morphology (microscopic). The macroscopic parameters observed included colony shape, edge, surface, and color. The microscopic parameters observed included cell shape and Gram staining properties.

The Gram staining properties of bacteria were determined by performing Gram staining to differentiate between Gram-positive and Gram-negative bacteria. Gram staining was performed by taking 10 µl of bacterial isolate and placing it on a microscope slide, followed by the application of Gram staining components consisting of Gram A (crystal violet), Gram B (lugol), Gram C (96% ethanol), and Gram D (safranin). The results of the Gram staining were then observed under a microscope at 100x magnification.

Physiological Characterization of Bacterial Isolates

Catalase Test, this test was performed by adding 3% H_2O_2 to the bacterial isolate. A positive result was

indicated by the formation of oxygen bubbles on the bacterial isolate treated with 3% H_2O_2. H_2S Production, performed by growing bacterial isolates on Sulfide Indole Motility (SIM) media. Each bacterial isolate was collected using an inoculating needle and then inoculated by stabbing the needle into the SIM media. A positive result was indicated by the formation of a black precipitate at the bottom of the tube.

Indole Test, performed by growing bacterial isolates on Sulfide Indole Motility (SIM) media and adding Kovac's reagent. A positive result was indicated by the formation of a red ring. Motility Test, performed by growing bacterial isolates on Sulfide Indole Motility (SIM) media. Each bacterial isolate was collected using an inoculating needle and then inoculated by stabbing the needle into the SIM media. A positive result was indicated by bacterial growth outside the stab line.

Cellulase Enzyme Activity Test

The cellulase enzyme activity test was conducted by growing selected pure isolates on 1% Carboxy Methyl Cellulose (CMC) media. After incubation, the plates were flooded with 0.1% Congo red solution and rinsed with 1M NaCl solution. The presence of cellulolytic activity was indicated by the formation of clear zones around the colonies. The cellulolytic index was then measured to quantify enzyme activity.

The enzyme activity test referred to the method by Bairagi et al. (2002) with slight modifications. The media used for the cellulase enzyme activity test consisted of 1 g sodium chloride, 1 g tryptone, 1.5 g agar, 0.5 g yeast extract, and 1 g carboxymethyl cellulose (CMC). The media was prepared in petri dishes with a volume of 20-25 ml, and paper discs were placed according to the number of inoculants tested. A 20 µl bacterial suspension (OD600: 0.25 ± 0.05) was added onto the paper discs, followed by incubation at 37 ^oC for 72 hours. Enzyme activity identification was performed by immersing the CMC agar media in 0.1% Congo red solution for 15 minutes and rinsing with 1M NaCl solution. The presence of enzyme activity was indicated by the formation of clear zones around the bacterial colonies. The cellulolytic index (CI) was calculated by subtracting the colony diameter (CD) from the diameter of the clear zone (DZ) and then dividing by the colony diameter (CD), as shown in Figure 2. Cellulose degradation capability was classified based on the cellulolytic index value: if the CI value was ≤ 1, it was considered low; if the CI value was 1-2, it was considered moderate; and if the CI value was ≥ 2, it was considered high (Choi et al., 2005). Formula for Cellulolytic Index is shown below (Choi et al., 2005).

$$\text{Cellulolytic Index (CI)} = \frac{\text{DZ} - \text{DC}}{\text{DC}} \quad (1)$$

Description:
DZ: Diameter of the clear zone
DC: Diameter of the colony

Data Analysis

The data analysis involved assessing the degradation capability of isolates based on the area of the clear zone and the morphology of bacterial colonies. This analysis was conducted descriptively and presented in tables and figures.

Result and Discussion

The isolation of bacteria is a crucial step to obtain pure bacterial isolates (single colonies) as intended. Ibrahim et al. (2015) explain that bacterial isolation is a technique used to obtain single colonies of bacteria from their natural environment and culture them on artificial media according to the isolation purpose. Bacterial isolation can be achieved using the spread plate method through serial dilution. The bacteria isolated in this study originated from the digestive tract of swans (*Cygnus olor*), including the proventriculus, small intestine (duodenum, jejunum, ileum), caecum, and colon.

The isolation results of bacteria sourced from the swan digestive tract on solid MRS media yielded a total of 8 pure bacterial strains. The bacterial isolates obtained include strains S_1, S_3, J, J_1, J_{21}, J_2, I_2, and UB_1. The bacterial isolation results on MRS agar media can be viewed in Figure 2.

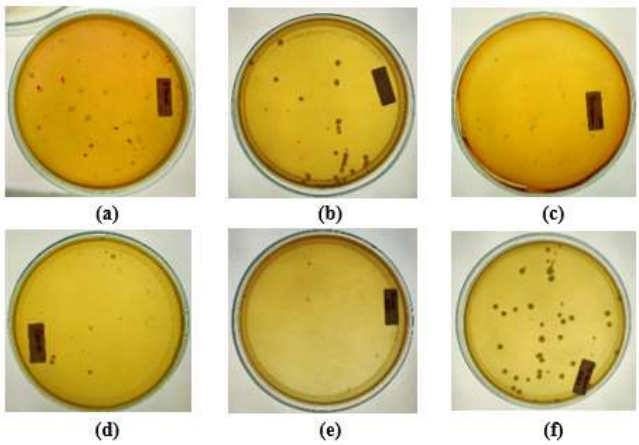

Figure 2. Bacterial Colonies from Various Parts of the Swan Digestive Tract with a 10-4 Dilution. a) Proventriculus; b) Duodenum; c) Jejunum; d) Ileum; e) Caecum; f) Colon

The bacterial cultures that grew were then purified based on differences in colony morphology using the streak plate method. Morphological observations of colonies were conducted with parameters such as colony

shape, margin, elevation, and color. The results obtained showed diverse outcomes as presented in Table 1.

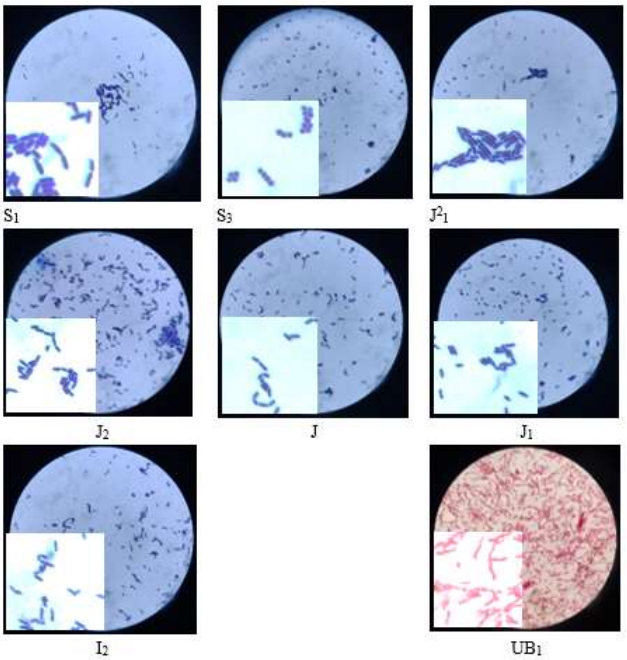

Figure 3. Representation of Gram Staining Results of Bacterial Isolates

Table 1. Macroscopic Characteristics of Bacterial Isolates

Isolate Code	Colony morphology			
	Shape	Elevation	Margin	Colour
S_1	Circular	Raised	Entire	White-cream
S_3	Circular	Raised	Entire	White-cream
J	Circular	Raised	Entire	White-cream
J_1	Circular	Raised	Entire	White-cream
J^2_1	Circular	Raised	Entire	White-cream
J_2	Circular	Raised	Entire	White-cream
I_2	Circular	Raised	Entire	White-cream
UB_1	Circular	Flat	Entire	White

Differences in morphology among species are one of the methods used to identify various bacteria. Based on Table 1, it can be observed that all eight isolates have circular colony shapes with smooth margins. The elevation of the isolates shows that seven isolates have convex elevations, while one isolate has a flat elevation. The color of the bacterial isolates is predominantly cream-white, with only one isolate being white.

Microscopic observation of bacterial isolates obtained from the swan digestive tract was conducted to determine the bacterial cell shape and Gram staining properties. Gram staining can differentiate between Gram-positive and Gram-negative bacteria due to differences in their cell wall structures. Gram-positive bacteria retain the purple color of crystal violet stain, which is absorbed and retained despite exposure to 96% ethanol. On the other hand, Gram-negative bacteria appear red because the crystal violet-iodine complex is dissolved by ethanol, allowing the red safranin or fuchsin counterstain to be absorbed (Lay, 1994). The ability of Gram-positive bacteria to retain the purple color is due to their cell wall structure containing approximately 10% teichoic acid and 90% peptidoglycan (Hadioetomo, 1993; Dewi, 2013; Detha, 2019; Zubaidah et al., 2022), whereas Gram-negative bacteria have cell wall structures with high lipid content (Fitri & Yasmin, 2011; Sabdaningsih et al., 2013; Yulvizar, 2013; Suarjana et al., 2017). Based on the data in Table 2, the Gram staining results show that out of the 8 bacterial isolates tested, 7 isolates are Gram-positive (+) and 1 isolate is Gram-negative (-).

Table 2. Microscopic Characteristics of Bacterial Isolates Based on Cell Shape and Gram Staining Properties

Isolate code	Cell Morphology	
	Cell Shape	Gram
S_1	Bacil	Positive
S_3	Coccus	Positive
J	Bacil	Positive
J_1	Bacil	Positive
J^2_1	Bacil	Positive
J_2	Bacil	Positive
I_2	Bacil	Positive
UB_1	Bacil	Negative

Physiological tests were conducted to obtain characteristic data from bacterial isolates isolated from the swan digestive tract. The physiological test results for the 7 bacterial isolates obtained are presented in Table 3. Based on the data in Table 3, all seven bacterial isolates showed negative catalase results, indicated by the absence of bubble formation after reacting with 3% H_2O_2. This finding is consistent with the studies by Laily et al. (2013), Hamidah et al. (2019), AlKalbani et al. (2019), Risna et al. (2020), and Anindita (2022), where lactic acid bacteria from the genera *Lactobacillus spp.*, *Pediococcus pentosaceus*, and Enterococcus were unable to produce catalase.

Next, motility, H_2S production, and indole tests were conducted by growing bacteria in SIM media. The SIM test results for the seven bacterial isolates showed that two bacterial isolates (J and I_2) did not produce H_2S gas, while the other five isolates (J_{21}, J_2, J_1, S_1, and S_3) did produce H_2S. Additionally, the indole production test resulted in negative findings for all eight isolates, as none of them formed a red ring. The motility test indicated that all eight bacterial isolates were motile, as evidenced by bacterial growth not only within the stab area but also spreading outside of it. Based on these test results, the bacterial isolates obtained are indicative of lactic acid bacteria (LAB) group. This aligns with research conducted by Tuo et al. (2013) and Yenni et al.

(2018) which states that bacteria exhibiting characteristics of Gram-positive, catalase-negative, non-motile, and coccus or bacillus shapes can be classified within the LAB group.

Table 3. Physiological Characteristics of Bacterial Isolates

Isolate code	Characteristic			
	Catalase	H_2S Production	Indol	Motility
S_1	-	+	-	+
S_3	-	+	-	-
J	-	-	-	+
J_1	-	+	-	+
J^2_1	-	+	-	+
J_2	-	+	-	+
I_2	-	-	-	+

Cellulase Enzyme Activity Test

Cellulase enzyme is an enzyme that accelerated the hydrolysis process of cellulose or substrates with beta bonds found in cellulose, thereby hydrolyzing polymers randomly and producing simple cellulose molecules. This enzyme was rarely expressed in the digestive tract of monogastric poultry, so the high fiber content in feed materials could not be maximally digested and often excreted directly through excreta, which could be a source of ammonia and environmental pollution. Seprianto (2017) explained that cellulase enzymes were commonly used to refine paper powder with high fiber content, thereby enhancing the quality of the food industry and as a fiber degradation agent in feed to improve nutrient content in feed.

Extracellular enzyme activity tests on several bacteria isolated from the digestive tract of swans (*Cygnus olor*) were conducted using Carboxy methyl cellulose (CMC) media, following the procedure outlined by Suryadi et al. (2023). The results of cellulase enzyme activity tests on each bacterium are presented in Figure 4.

The results of the cellulase enzyme activity test showed that out of the 7 bacterial isolates tested, 4 bacterial isolates were capable of producing cellulase enzymes, as indicated by the presence of clear zones around bacterial colonies after being soaked in 0.1% red congo solution and washed with NaCl solution. Some of the isolates included (S_1, J_1, J_{21}, and I_2). Meanwhile, the remaining 3 isolates did not show any cellulase enzyme activity, identified by the isolate codes (S_3, J, and J_2), and it is possible that they produce other enzymes which require further testing to confirm their identity. Among the cellulase-positive bacteria, it can be concluded that they have the ability to produce different enzymes, as evidenced by the diameter of the clear zones produced. The area of the clear zones from the enzyme activity test is presented in Table 4 below.

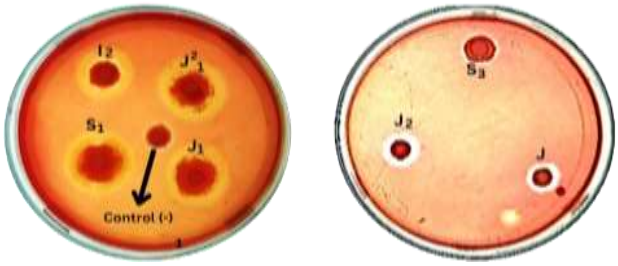

Figure 4. (a) Cellulase enzyme activity indicated by the formation of clear zones and there is a negative control with isolate code x; (b) No cellulase enzyme activity

Table 4. Area of Clear Zones from Cellulase Enzyme Activity Test

Isolate code	Clear zone diameter (mm)	Colony diameter (mm)	Cellulotic index
S_1	27	16	0,69
J_1	24	16	0,5
I_2	19	13	0,46
J_{21}	20	14	0,43

Based on the above data, the bacterial isolate with code S_1 exhibited the best ability to produce cellulase enzyme with a cellulolytic index value of 0.69. Following that, bacterial isolate J_1 showed cellulase enzyme production capability with a cellulolytic index of 0.5, bacterial isolate I_2 had cellulase enzyme production capability with a cellulolytic index of 0.46, and bacterial isolate J_{21} exhibited the lowest cellulase enzyme production capability with a cellulolytic index of 0.43. All bacteria obtained in this study belonged to the low category as they had cellulolytic index values ≤ 1. It is predicted that cellulolytic bacteria exist in the digestive tract of ducks due to the influence of their diet containing fiber such as vegetables, bran, and grasses.

Conclusion

Based on the research conducted, four bacterial isolates identified as lactic acid bacteria (LAB) exhibited cellulolytic activity with varying capabilities. The bacterium showing the highest cellulolytic activity was isolate S_1 (from the caecum), which had a cellulolytic index of 0.69. Meanwhile, the lowest cellulolytic activity was observed in isolate J_{21} (from the jejunum), with a cellulolytic index of 0.43.

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Author Contributions
Conceptualization and investigation, M. S. B. R; Methodology and data curation, M. S. B. R; Writing-review and editing, M. S. B. R, K, Q. A. S; supervision, M. A, I. W. W, D. K.

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Conflicts of Interest
The authors declare no conflict of interest.

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