Research Article

Isolation and Characterization of Chitinolytic Lactic Acid Bacteria from Shrimp Waste as a Potential Source of Chitinase

Maida Elisa Solta[1], Mirzah Mirzah[2]*, Mirnawati Mirnawati[2], Anthoni Agustien[3]

[1]Faculty of Animal Science, Universitas Andalas, Padang, West Sumatera, Indonesia. [2]Department of Animal Feed and Nutrition, Faculty of Animal Science, Universitas Andalas, Padang, West Sumatera, Indonesia; [3]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Andalas, Padang, West Sumatera, Indonesia.

Abstract | The increased use of microbial enzymes in agriculture and animal husbandry requires the development of safe and sustainable biological conversion technologies for the utilization of alternative feed materials. Shrimp waste produces chitin-rich material, but its utilization is still limited due to the rigid structure of chitin and its insolubility in water. Microorganisms that produce chitinase play an important role in the bioconversion of waste. This study aimed to isolate, screen, and identify chitinolytic lactic acid bacteria (LAB) from shrimp waste as a new source of chitinase. A total of three LAB isolates were obtained, all of which showed chitin hydrolysis zones after 96 hours of incubation on chitin-based selective media. The chitinolytic index ranged from 1.04 to 1.24, with isolate M1 showing the highest hydrolysis power. Chitinase activity, measured using the dinitrosalicylic acid (DNS) method, ranged from 0.103 to 0.106 U/mL, with isolate M1 exhibiting the highest activity. Based on morphological characterization, Gram staining, and biochemical tests, the three isolates were identified as *Pediococcus* sp. 1 (M1), *Pediococcus* sp. 2 (M2), and *Pediococcus* sp. 4 (M4). These results indicate that LAB from shrimp waste have potential to be used as an environmentally friendly fermentation starter in the bioconversion of chitin-based feed materials to improve poultry feed digestibility.

Keywords | Chitinase activity, Chitinolytic, Isolation, Lactic acid bacteria, Shrimp waste

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***Correspondence** | Mirzah Mirzah, Department of Animal Feed and Nutrition, Faculty of Animal Science, Universitas Andalas, Padang, West Sumatera, Indonesia; **Email:** mirzah@ansci.unand.ac.id
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INTRODUCTION

The use of microbial enzymes in industries such as food, agriculture, chemistry, and pharmaceuticals has experienced rapid growth and attracted greater interest than enzymes derived from plants and animals (Singh *et al*., 2016). Waste can be an important source of bacterial and enzyme diversity with potential for biotechnology applications to be utilized as livestock feed (Muwakhid *et al*., 2025). Microbial bacteria and enzymes can be used to address environmental problems arising from industrial activities (Singh *et al*., 2016). One industry that needs strategies to reduce the environmental impact of waste is the shrimp aquaculture industry (Páez-Osuna, 2001).

Shrimp production in aquaculture provides a rich source of protein for humans (Kandra *et al*., 2012). However, this activity produces large amounts of shrimp shell waste (Dai *et al*., 2015). Shrimp waste consists of 45.40% protein, 11.17% calcium, 6.86% sodium, 237 mg/kg iron, 77 mg/kg zinc (Jiménez-Gomez *et al*., 2024), and 10–40% fat (Tan *et al*., 2020). In addition, shrimp waste also contains about

20–30% chitin (Filawati *et al.*, 2018) that has the potential to be used as poultry feed.

Among these various components, chitin is a natural biopolymer with high availability and ranks second after cellulose. Chitin has a stable, rigid, and water-insoluble crystalline structure due to the N-acetylglucosamine polymer chains that form microfibrils through intermolecular hydrogen bonds (Elieh-Ali-Komi and Hamblin, 2016; Dliyauddin *et al.*, 2020). To overcome these limitations, the use of chitinase enzymes is a promising approach. Chitinase enzymes play an important role in various aspects of biotechnology, such as the biological conversion of chitin waste, biological control of pathogenic fungi, production of organic fertilizers, and increasing the nutritional value of fibrous feed materials (Taokaew *et al.*, 2023).

Various studies have reported the existence of chitinolytic bacteria capable of degrading shrimp shell waste (Setia, 2015). However, most previous studies have focused on chitinolytic bacteria. Research on lactic acid bacteria (LAB) as producers of chitinase enzymes is still limited and under-explored. LAB are Gram-positive, non-spore-forming bacteria that are facultative anaerobes and capable of fermenting carbohydrates, primarily into lactic acid (Ruiz *et al.*, 2019). These bacteria are generally classified as Generally Recognized As Safe (GRAS) and are widely used as probiotics and fermentation starters in various food and feed applications (Vieco-Saiz *et al.*, 2019). The objective of this study was to isolate and identify chitinolytic LAB from shrimp waste and evaluate its potential as a new source of chitinase enzyme to support safe and sustainable biological conversion technology for poultry feed.

MATERIALS AND METHODS

Research design

This research was conducted in a laboratory through several stages. The first step was to isolate LAB on the Man Rogosa and Sharpe (MRS) agar (Merck, Germany). After that, LAB was tested for its ability to produce chitinase enzyme on colloidal chitin agar (CCA) medium. The isolate obtained were then tested for enzyme activity. The isolates were then selected based on their morphological characteristics.

Isolation and screening of lactic acid bacteria

This study began with the decomposition process of shrimp waste. After that, isolation was carried out aseptically, 1 ml of shrimp waste was suspended in a 0.85% NaCl solution. The liquid suspension was then inoculated into isolation media on MRS Agar. It was then incubated for 24 hours at a temperature of 37 °C. Further purification was carried out by etching on MRS Agar supplemented with 1% (w/v) $CaCO_3$ was incubated at 37 °C for 24 hours. The clear zone on the Petri dish indicated that the bacteria belonged to the lactic acid bacteria group.

Chitinolytic LAB screening

Bacteria were isolated to obtain chitinase-producing strains. The LAB isolates were subsequently evaluated for their ability to produce chitinase using a selective medium prepared according to Saima *et al.* (2013). The medium contained (g/L): 1% colloidal chitin (w/v), 6.0 Na_2HPO_4 (Merck, Germany), 1.0 K_2HPO_4 (Merck, Germany), 0.5 NH_4Cl (Merck, Germany), 3.0 KH_2PO_4 (Merck, Germany), 0.12 $MgSO_4 \cdot 7H_2O$ (Merck, Germany), 0.5 NaCl (Merck, Germany), 0.05 yeast extract (Himedia, India), and 15 agar, adjusted to pH 7. The medium was autoclaved at 121°C for 15 min and incubated at 37°C for 4 days (96 h) to allow chitinolytic activity (Sudha *et al.*, 2020).

Preparation of colloidal chitin

Colloidal chitin (CC) was prepared from chitin powder by the method of Monreal and Reese (1969) with minor modifications. Chitin powder (30 grams) was slowly added to 300 mL of concentrated HCl and kept overnight at 4 °C. The suspension was mixed with 1200 mL of cold ethanol (96%) with vigorous stirring and kept overnight at 4 °C. The precipitate was collected by centrifugation at 9000 rpm for 10 minutes, washed several times with distilled water until the colloidal chitin became neutral (pH 7.0). Final precipitate was dried to a constant weight at 50 °C and stored at room temperature until further use.

Production of crude chitinase enzyme extract

Bacteria were cultured in liquid media containing: 4.0 g/L yeast extract; 2.0 g/L tryptone; 4.0 g/L $MgSO_4.7H_2O$; 1.2 g/L KH_2PO_4; 2.8 g/L K_2HPO_4; and 15 g/L neutral chitin used as an inducer (Yamaguchi, 2003). The fermentation process was carried out in a 500 mL Erlenmeyer flask containing 100 mL of medium, then incubated at 25°C with a shaking speed of 200 rpm for 72 hours. After that, 10 mL of the fermentation product was transferred to a new flask containing 90 mL of the same medium, then incubated again under similar conditions for 72 hours. After incubation, the culture was centrifuged at 7,840 × g for 10 minutes at 5 °C. The supernatant (the liquid portion on top) was collected and used as crude enzyme for chitinase activity testing.

Chitinase enzyme activity

Chitinase activity was measured using the 3,5-dinitrosalicylic acid (DNS) method, using colloidal chitin as a substrate, according to the protocol described by Divarta *et al.* (2016), with slight modifications. The

enzyme solution (1.0 mL) was allowed to react with 1.0 mL of 0.5% colloidal chitin solution formulated in 0.1 M citrate buffer (pH 7.0) for 30 minutes. The mixture was allowed to react with 1.0 mL of 0.5% colloidal chitin solution, formulated in 0.1 M citrate buffer (pH 7.0), for 30 minutes. The reaction mixture was incubated at 37°C in a shaking water bath. After incubation, the reaction was stopped by adding 2 mL of DNS reagent and heating the mixture for 10 minutes in a boiling water bath. The mixture was cooled and centrifuged at room temperature for 10 minutes at 10,000 rpm, and the absorbance of the supernatant was measured at 540 nm relative to the control. One unit of chitinase activity is defined as the amount of enzyme required to release reducing sugar at a rate of one milligram per minute per milliliter of enzyme solution. Activity is defined as the amount of enzyme required to release reducing sugar at a rate of one milligram per minute per milliliter of enzyme solution.

Characterization of LAB colonies

Gram staining

Gram staining begins with the preparation of the smear, namely by cleaning the slide with a piece of cotton soaked in alcohol, shaking the tube containing the bacterial suspension, taking one eye loop of the suspension and moving it to the center of the slide and smearing it and then letting it dry in the air for a while. The preparation was then fixed on a bunsen to kill and attach bacteria to the glass slide, dipped with ammonium oxalate violet, washed with running water, then given Lugol's iodine solution for one minute, rinsed with water, then given acetone solution for 10 seconds, then washed again. with running water. After that, the preparations were given carbol fuchsin solution for one minute and washed again with running water and then dried. The test was carried out at 1000 X magnification (Sunatmo, 2009). Observations were taken on the morphology of bacteria and gram properties (bluish purple color refers to gram-positive bacteria, while red or pink color refers to gram-negative bacteria).

Catalase test

The catalase test was performed by placing a drop of 3% hydrogen peroxide (H_2O_2) onto a sterile glass slide. A small amount of bacterial culture was then transferred using a sterile inoculating loop and mixed with the H_2O_2 on the slide. The formation of bubbles was observed as an indication of catalase activity. If bubbles did not occur, it meant that the bacteria were catalase negative, but if bubbles formed, it meant that the bacteria were catalase positive (Sunatmo, 2009).

Oxidase test

The oxidase test was performed by placing a few drops of oxidase reagent onto sterile filter paper. Then the bacteria were taken using a sterile inoculating loop and then homogenized on filter paper moistened with oxidase reagent. After that it was observed, if a blue color was not formed, it meant that the bacteria were oxidase negative, but if a blue color was formed, it meant that the bacteria were oxidase positive (Sunatmo, 2009).

Carbohydrate test

This test was done to find out whether the bacteria ferment each of the above sugars to form acid. This sugar medium was separated into 5 different tubes and the media used were each sugar with a concentration of 1% in peptone. Each sugar added indicator phenol red. Interpretation of results: Negative (-) if the media does not change color from red to yellow, meaning the bacteria do not ferment sugar. Positive (+) if there is a change in the color of the medium from red to yellow. This means that the bacteria fermenting the sugar are marked on the different cotton caps. For colorless glucose, lactose is purple, maltose is red, mannitol is green and sucrose is blue. In the sugar-acid medium, positive + gas (+g): the color of the medium changes from red to yellow. This means that bacteria ferment sugars to form acids and gases. The calculated gas is at least 100% of the test tube height (Cowan and Steel, 1974).

VP test (Voges-Proskauer)

This test was carried out by means of MR-VP media made in peptone in a tube, bacteria were inoculated using aseptic technique and incubated for 24-48 hours at 37°C. Observation was carried out by adding Barrit's A and B reagents. This test aimed to see the ability of isolate in releasing non-acidic substances or neutral end products such as acetylmethylcarbinol from organic acids as glucose metabolism.

Statistical analysis

This study used a descriptive design method. Chitinase enzyme activity was analyzed using One-Way ANOVA with six replications. Tuckey test at a confidence level of 0.05 ($P<0.05$) was used to see the significant differences among samples.

RESULTS AND DISCUSSION

Isolation and screening of lactic acid bacteria

The results showed that isolates obtained from shrimp waste could grow on MRS media supplemented with 1% $CaCO_3$ after incubation for 24 hours at 37 °C. The addition of $CaCO_3$ (1%) was used to enhance LAB selection, as indicated by the formation of clear zones. There were 12 LAB isolates isolated from shrimp waste, and 3 isolates showed chitinolytic LAB. The presence of clear zones confirmed that these isolates had the ability to produce lactic acid (Figure 1). The successful isolation of LAB

from shrimp waste indicates that the substrate provides a supportive environment for the growth of lactic acid-producing bacteria.

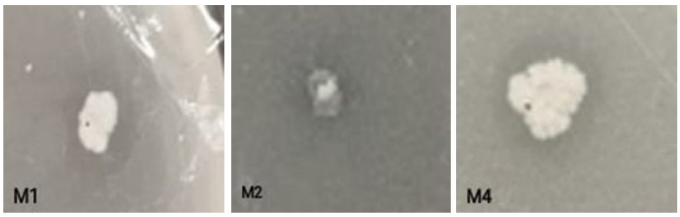

Figure 1: Clear zones in bacterial isolates M1, M2, and M4.

The protein, carbohydrate, and other organic matter in shrimp waste serve as a nutrient source that can be utilized by LAB during the fermentation process. Other studies have also reported that LAB can grow predominantly in fermented fishery by-products due to their ability to rapidly ferment carbohydrates and produce lactic acid as the primary metabolite (Kim *et al.*, 2019).

Chitinolytic LAB screening

Semi-quantitative analysis of chitinolytic bacterial activity was performed by measuring the clear zone around the colonies. The formation of a clear zone around the petri dish on selective medium indicated that LAB was chitinolytic (Figure 1). The three selected LAB isolates with the highest chitinolytic activity are presented in Table 1.

Table 1: Clear zone and chitinolytic index of LAB isolates isolated from shrimp waste.

Isolate code	Clear zone (mm)	Chitinolytic index
M1	8.5	1.24
M2	6.8	1.18
M4	3.02	1.04

Note: The clear zone represents the area formed due to chitin degradation. The chitinolytic index was calculated as the ratio of the diameter of the clear zone to the diameter of the bacterial colony.

A screening test was conducted to determine the ability of candidate bacteria to produce chitinase enzymes. Figure 1 shows clear zones in isolates M1, M2, and M4. The diameter of the LAB clear zone in this study ranged from 3.02 to 8.5 mm (Table 1). The clear zone around the colony indicates chitinase activity to break down chitin compounds in the medium (Zarei *et al.*, 2012). Among all isolates, M1 showed the largest clear zone, indicating stronger chitinolytic activity. Increased chitinolytic activity is influenced by the growth phase of the bacteria, the availability of substrates such as chitin oligomers, and the physiological characteristics of the bacterial species (Daulagala, 2017; Le *et al.*, 2018; El-Sayed *et al.*, 2018). The chitinolytic index values in this study were higher than those of chitinolytic bacterial isolates from shrimp rusip, with a range of 1.06–3.66 (Puspita *et al.*, 2017). This difference is related to the different ecological conditions of the isolate sources, where bacteria from fresh shrimp waste have a higher chitin degradation capacity than isolates from further fermentation products. Shrimp shell waste is a natural reservoir with higher concentrations of chitinolytic bacteria and enzymatic potential than other sources such as soil, sediment, or fermented food (Masri *et al.*, 2021).

Chitinolytic bacteria that produce clear zones demonstrate the ability to hydrolyze β- (1→4)-glycosidic bonds in chitin polymers, thereby degrading the chitin structure into simpler molecules. This chitinolytic activity is mediated by three main enzyme groups, namely endochitinase, exoglycosidase, and N-acetylglucosaminidase, which work synergistically in converting chitin into chitooligosaccharides and N-acetylglucosamine (Wibowo *et al.*, 2017).

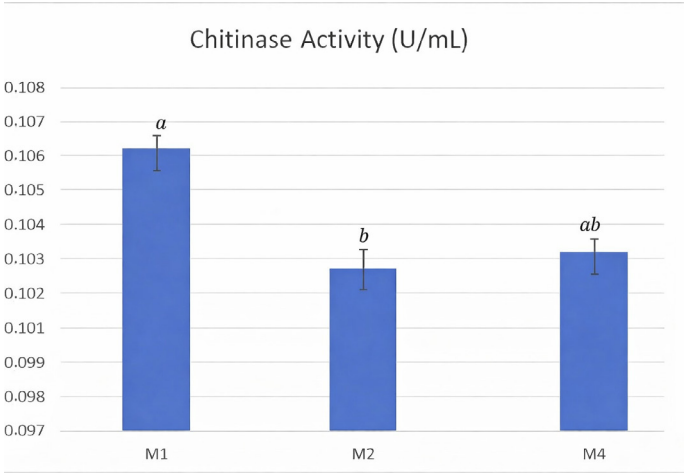

Figure 2: Chitinase enzyme activity of bacterial isolates M1, M2, and M4.

Chitinase enzyme activity

Based on the results of this study, chitinase activity in the three bacterial isolates ranged from 0.103 to 0.106 U/mL (Figure 2). Specifically, isolates M1, M2, and M4 exhibited activities of 0.106, 0.103, and 0.104 U/mL, respectively (Figure 2). The best results were obtained from isolate M1. Rathore and Gupta (2015) stated that chitinase activity is directly correlated with the amount of N-acetylglucosamine (GlcNAc) produced during chitin hydrolysis, so that measurement of enzymatic activity is considered a valid quantitative indicator of chitin degradation ability. Differences in enzyme activity in bacterial isolates may be due to differences in the bacterial strains used. Bacteria with different strains will produce different enzyme activities. This is in line with the opinion of Rietl *et al.* (2016), who stated that differences in microbial communities can produce

diverse enzymatic patterns, thus giving rise to variations in enzyme activity between microorganisms. Some microbes are capable of producing high enzyme activity, while others show low activity (Yi *et al.*, 2019). Therefore, the bacterial species used can affect the enzyme activity produced. The chitinase activity obtained in this study was higher than that of isolates from shrimp waste, which only produced 0.02–0.05 U/mL (Puspita *et al.*, 2017).

Biochemical characteristics of lactic acid bacteria

The morphological characteristics of LAB isolates are presented in Table 2. All three isolates (M1, M2, and M4) exhibited similar characteristics consistent with those of lactic acid bacteria. Gram staining is a technique that can distinguish bacteria into two groups, namely Gram-positive and Gram-negative (Yanestria *et al.*, 2019). The Gram staining reaction of lactic acid bacteria colonies is shown in Table 3. All colony samples tested showed Gram-positive results under a microscope. The Gram staining results of the identified lactic acid bacteria species showed characteristics including purple color, cocci shape, and size (short and medium) that are included in the gram-positive bacteria group. The shape of all isolated lactic acid bacteria species was chain-like cocci, clustered and arranged singly, and the purple color indicated that the isolated species were gram-positive.

Table 2: Morphological characteristics of LAB isolates from shrimp waste.

Morphology	Isolate code		
	M1	M2	M4
Colony shape	Circular	Circular	Circular
Shape	Cocci	Cocci	Cocci
Colony color	Milky white	Milky white	Milky white
Elevation	Convex	Convex	Convex
Edge	Entire	Entire	Entire

Note: Morphological characteristics were observed based on colony shape, cell shape, color, elevation, and edges on selective agar medium.

Gram-negative bacteria have a thinner peptidoglycan layer than Gram-positive bacteria (Somani *et al.*, 2023). Gram-positive bacteria have a peptidoglycan layer 30–100 nm thick or more, while Gram-negative bacteria only have a layer of a few nanometers (Rohde *et al.*, 2018). The Gram staining results and microscopic observations from this study are consistent with similar results reported by Asnake and Mogessie (2010), Tamene *et al.* (2019), and Tilahun *et al.* (2018).

In this study, all identified LAB species were tested for their catalase reaction. The results showed that all identified LAB isolates exhibited a negative catalase reaction when H_2O_2 was dropped onto fresh cell cultures on microscope slides (Table 3). A positive catalase reaction occurs when air bubbles appear, indicating the formation of O_2 gas, and a negative reaction occurs when air bubbles are formed, indicating the absence of gas bubbles, which is one of the characteristics of lactic acid bacteria (Mullaw *et al.*, 2019). The results obtained in this study indicate that the identified LAB isolates are unable to produce the catalase enzyme to convert hydrogen peroxide into water and oxygen. These results are in line with those reported by (Desiye and Abegaz, 2013; Mullaw *et al.*, 2019; Tafesee *et al.*, 2020).

Table 3: Biochemical characteristics of LAB isolates from shrimp waste.

Characteristics	Isolate		
	M1	M2	M4
Gram Staining			
Shape	Cocci	Cocci	Cocci
Grams	+	+	+
Spore	-	-	-
Biochemical test			
Aerobic/anaerobic	Aerobic	Aerobic	Aerobic
TSIA	b/b	b/b	b/b
H_2S	-	-	-
Catalase	-	-	-
Oxidase	-	-	-
Citrate	+	+	-
Indole	Non motile	Non motile	Non motile
Urease	+	+	±
Voges Proskauer (VP)	-	-	-
Sugar fermentation			
Glucose	+	+	+
Lactose	+	+	+
Maltose	+	+	+
Mannitol	+	+	±
Sucrose	+	+	+
Dulcitol	-	-	-
Galactose	±	±	+
Arabinose	±	±	±
Xylose	±	±	±
Rhamnose	-	-	-
Sorbitol	-	-	-
Raffinose	-	-	-
Salicin	+	+	-
Trehalose	±	±	+
Inositol	±	±	-
Cellobiose	+	+	+
Aesculin	+	+	+
Strains	*Pediococcus sp.* 1	*Pediococcus* sp. 2	*Pediococcus* sp. 4

Note: Morphological characteristics of *Pediococcus* were determined according to Bergey's Manual of Systematic Bacteriology. (+): Positive, (-): Negative (±): Weak reaction.

Based on the examination of colony morphology, cell morphology, biochemical tests, and sugar fermentation assays, the characteristics of the isolate obtained were consistent with the genus *Pediococcus*. *Pediococcus* is a group of lactic acid bacteria (LAB) characterized as Gram-positive, catalase-negative, facultatively anaerobic, homofermentative, non-motile, and non-spore-forming organisms, and they belong to the family *Lactobacillaceae* within the order *Lactobacillales* (Wieme *et al*., 2012; Holzapfel *et al*., 2015).

CONCLUSION

In this study, three lactic acid bacteria isolates were found to produce chitinase enzymes. Isolate M1 had the highest chitinolytic activity. All isolates M1, M2, and M4 had characteristics similar to bacteria of the genus Pediococcus. Where isolate M1 was categorized as *Pediococcus* sp. 1, isolate M2 was categorized as *Pediococcus* sp. 2, and isolate M4 was categorized as *Pediococcus* sp. 4.

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NOVELTY STATEMENT

This study identified shrimp waste as a novel source of lactic acid bacteria isolates acting as chitin fermentation agents, which provides a sustainable microbial-based approach for waste bioconversion and poultry feed quality improvement.

AUTHOR'S CONTRIBUTION

Mirzah, Mirnawati, and Anthoni Agustien developed the script concept. Maida Elisa Solta conducted experiments, analyzed data and wrote a script.

ETHICS APPROVAL

This study does not require ethics approval because we did not use animals, but instead used lactic acid bacteria isolates from shrimp waste.

GENERATIVE AI AND AI ASSISTED TECHNOLOGY STATEMENT

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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