P-ISSN: 2304-3075; E-ISSN: 2305-4360
International Journal of Veterinary Science
www.ijvets.com; editor@ijvets.com

Research Article https://doi.org/10.47278/journal.ijvs/2023.067

Isolation and Characterization of Cellulolytic Lactic Acid Bacteria from Soymilk Waste as Probiotic Candidates for Broiler

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Article History: 23-171 Received: 08-Apr-23 Revised: 04-May-23 Accepted: 09-May-23

ABSTRACT

Lactic acid bacteria (LAB) have the potential to be used as probiotics for broilers because they have the ability to release enzymes, one of those is cellulase enzyme. However, the lactic acid bacteria are still unknown; thus, they need to be found. This study aims to find cellulolytic LAB from soymilk waste as a probiotic candidate for broiler chickens. This research was conducted through several stages, those were isolation of LAB, LAB bacteria were then tested for their ability to produce cellulase enzyme. After that, the isolates were tested for their enzyme activity and the isolates obtained were then selected for their morphological characters. The results showed that the isolates found namely F4, F6, F9 and F11 were cellulolytic lactic acid bacteria. These four isolates are classified as gram-positive bacteria and are aerobic. The cellulase activities of isolates F4, F6, F9 and F11 were 17.69, 20.67, 14.72 and 13.13U/mL, respectively. Based on the characterization of the bacteria, isolates F4 and F6 were categorized as *Lactobacillus* sp.1, isolate F9 was marked as *Lactobacillus* sp.2 and isolate F11 was characterized as *Lactobacillus* sp.3.

Key words: Isolation, Characterization, Cellulolytic, Lactic Acid Bacteria

INTRODUCTION

Lactic acid bacteria (LAB) are a type of bacteria that are usually found in milk, plants, fermented milk products and vegetables, as well as the digestive tract of humans and animals, water and soil (Leska et al. 2022). LAB are facultative anaerobes, acid tolerant, non-sporulating, gram-positive microorganisms with rod (*bacilli*) or round (*cocci*) shapes, arranged in pairs or in chains, immobile, non-sporulating, non-motile and mesophilic (Ray 2004). LAB can release lactic acid as the end product of carbohydrate fermentation (Ruiz et al. 2019). LAB can produce several active metabolites such as hydrogen peroxide, ethanol and bacteriocin. LAB is Generally Recognized As Safe (GRAS) (Sadiq et al. 2019) so it can be used as a probiotic agent. The most common types of microorganisms used as probiotics are lactic acid bacteria such as *Lactobacillus spp*., *Bacillus spp.*, *Lactococcus spp*., *Bifidobacterium spp*. and *Streptococcus spp*. (Park et al. 2016; Khyralla et al. 2022).

LAB isolation is mostly done to obtain bacteria that can be used as probiotics in livestock. LAB is used as a probiotic because of its ability to modify the environment by producing different metabolites including various inhibitory and competitive substances (Gaggìa et al. 2010; Gul et al. 2023). The substances produced include organic acids, bacteriocins, ethanol, diacetyl, carbon dioxide and H_2O_2 (Liao and Nyachoti 2017). The lactic acid released by LAB raises poor local microenvironment for pathogenic bacteria (Dittoe et al. 2018). However, this acid does not influence the epithelial cells of livestock because of a pH gradient that is created by mucus layer (Allen and Flemström 2005). LAB is also known to produce cellulase enzymes which can help digest crude fiber, for example *Bacillus velezensis* (Li et al. 2020). The problem with various feed ingredients in chickens is the presence of a limiting factor in the form of high cellulose. Poultry have limited ability to digest cellulose. Therefore, it is necessary to give probiotics derived from LAB which are cellulolytic to overcome this problem.

Cite This Article as: Srifani A, Mirnawati, Marlida Y, Rizal Y and Nurmiati, 2023. Isolation and characterization of cellulolytic lactic acid bacteria from soymilk waste as probiotic candidates for broiler. International Journal of Veterinary Science x(x): xxxx. https://doi.org/10.47278/journal.ijvs/2023.067

Cellulolytic bacteria have the ability to degrade substrates containing cellulose. These bacteria obtain carbon and energy sources by altering cellulose into sugar and used them for metabolism and growth (Vlasova et al. 2016). Cellulolytic bacteria have an advantage over other microorganisms such as fungi because they are able to grow faster so it takes less time to produce enzymes (Barzkar et al. 2020; Moseri et al. 2023).

Microorganisms that have suitable qualities can be isolated from natural environments, where basic natural substances affect the metabolism of microorganisms and direct their metabolic pathways to produce the enzymes needed (Lata et al. 2013). Therefore, cellulolytic lactic acid bacteria can be isolated from materials that contain high crude fiber, such as soymilk waste which have a crude fiber content of 18.15% (Cipta et al. 2021). However, isolates of cellulolytic LAB as probiotic candidates for broiler chickens have not yet been found. Based on this background, this study aimed to find cellulolytic LAB isolates from soymilk waste as probiotic candidates.

MATERIALS AND METHODS

Ethical Approval

This research did not require ethical approval because we did not use animals but instead used isolates of lactic acid bacteria those were isolated from soymilk waste.

Study Period and Location

This study was organized from August - November 2022 at the Feed Industry Technology Laboratory, Faculty of Animal Husbandry, Andalas University (Padang, Indonesia) and Bukittingi Veterinary Center (Bukittinggi, Indonesia).

Research design

This research was established in the laboratory in several stages. The first step was to isolate the LAB on De Man Rogosa and Sharpe (MRS) agar (Merck, Germany). After that, LAB bacteria were tested for their ability to produce cellulase enzymes on CMC media. The isolates were then tested for its enzyme activity. The isolates obtained were then selected for morphological characteristics.

Isolation and Screening of Lactic Acid Bacteria

This research started from the souring process of soymilk waste. After that, the isolation was carried out aseptically, 1gram of soymilk waste was suspended in 100mL of sterile distilled water. The liquid suspension was then embedded in the isolation medium on MRS Agar. It was then incubated for 24 hours at 37°C. Further purification was carried out by etching on MRS Agar which had added $CaCO_3$ 1 % and incubated at 37°C for 24 hours. The clear zone on the petri dish indicates that the bacteria are classified as lactic acid bacteria.

Cellulolytic LAB Screening

Bacterial isolation was carried out to find cellulose-producing bacteria using the Bergeys Manual method (1986). The LAB isolates obtained were then tested for their ability to produce cellulase enzymes using CMC media with a composition of 0.02g $MgSO_4 .7H_2O$; 0.075g KNO_3; 1g CMC, 0.05g K_2HPO_4; 0.002g $FeSO_4$; 0.004g $CaCl_2$; 0.2g bacterial agar and 0.1g glucose in 100mL of distilled water. The media was then autoclaved for 15min at 121°C. The media was poured into petri dishes and scratched with bacteria isolates. It was then incubated at 30°C for 24 hours and observed for the emersion of clear zone. The plates were stained with 0.1% (w/v) Congo Red dye for 15min and then washed with 1M NaCl solution for 15-20min. The unstained area (hydrolyzed clear zone) indicates the location where CMC was hydrolyzed. Cellulolytic isolates were selected based on the hydrolysis zone surrounding the colony.

Cellulase Enzyme Activity

Cellulase enzyme activity was measured based on the method of Jennifer and Thirunelakandan (2015). One mL of the enzyme supernatant was added to 1mL of extract (0.5mL CMC + 10mL of acetate buffer), then incubated for 30min at 40°C in a water bath shaker, 1mL was taken and added to 1mL of nelson AB, then heated in boiling water for 20min, after cold it was then added with 1mL of phospomolybdate and 7mL of distilled water, then the absorbance was read at 575nm. To see the magnitude of cellulase activity, the following formula was used:

$$Aktifitas\ sellulase\ \left(\frac{U}{ml}\right) = \frac{X\ x\ P\ x\ 1000}{t\ x\ BM}$$

Note: X = standard curve conversion result
P = Dilution
T = Time
BM = Glucose Molecular Weight

Characterization of LAB Colonies

a. Gram staining (Sunatmo 2009)

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, dripped 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. 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).

b. Catalase Test (Sunatmo 2009)

The catalase test was carried out by dripping H_2O_2 on a sterile glass object, then the bacteria were taken using a sterile loop needle, then homogenized in the H_2O_2 liquid on a sterile glass object. After that it was observed, 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.

c. Oxidase Test (Sunatmo 2009)

The oxidase test was carried out by dripping the oxidase reagent on sterile filter paper. Then the bacteria were taken using a sterile ose needle and then homogenized on filter paper dripped 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.

d. Carbohydrate Test (Adam 2001)

This test is 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.

e. Nitrate Reduction Test

This test was carried out aiming to know the ability of bacteria to degrade to reduce nitrate (NO_3) to nitrite (NO_2). This test was carried out by adding 0.1% KNO_3 as a nitrate substrate to the bacterial nutrient medium. The bacteria were inoculated and incubated for 24 - 48 hours at 37°C, after the incubation was completed reagent A (sulfanilic acid and reagent B (alphanafphylamine) were added to cause a color change.

f. 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. Cellulase enzyme activity was analyzed using One-Way ANOVA with three replications. Tuckey test at a confidence level of 0.05 ($P<0.05$) was used to see the difference in each sample.

RESULTS

Isolation and Screening of Lactic Acid Bacteria

A total of 4 isolates were obtained from soymilk waste in media containing MRS agar and $CaCO_3$. Each isolate produced different clear zones (Fig. 1) which indicated that the isolate had the ability to produce lactic acid.

Cellulolytic LAB isolation

A semi-quantitative analysis of cellulolytic bacterial activity was performed by measuring the clear zones around the colony. The formation of a clear zone around the petri dish on MRS agar plus CMC indicates that LAB are cellulolytic.

Cellulase Enzyme Activity

Cellulase enzyme activity of the 4 bacterial isolates found is shown in Fig. 2.

Characterization of Cellulolytic LAB Isolates

Biochemical tests for 4 bacterial isolates are shown in Table 3.

DISCUSSION

Isolation and Screening of LAB

The data presented in Fig. 1 showed that 4 lactic acid bacteria isolates were successfully isolated from soymilk waste. A total of 4 isolates were able to grow on

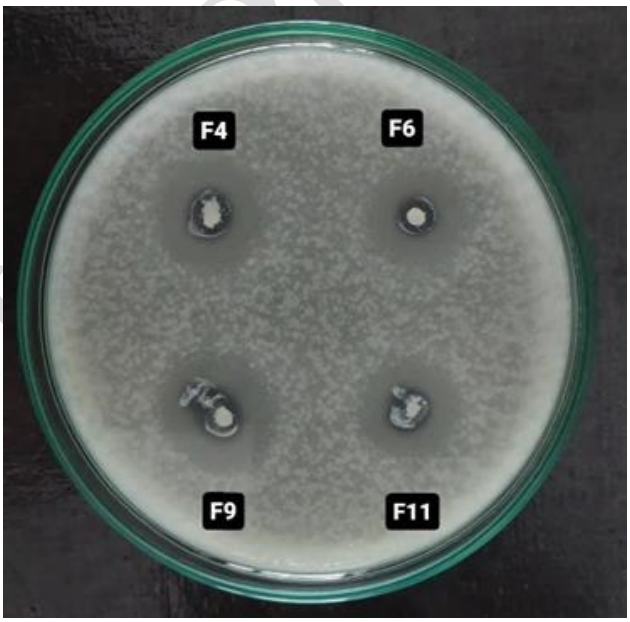

Fig. 1: Screening of lactic acid bacteria.

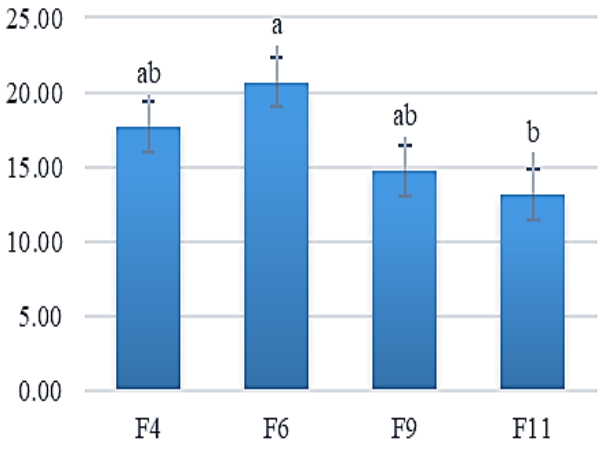

Fig. 2: Cellulase enzyme activity (U/mL) of LAB isolates.

MRSA+1% $CaCO_3$ media and showed a clear zone around the colony. This showed the capability of bacteria to grow and dissolve $CaCO_3$ in the medium (Mahulette et al. 2016). The appearance of the halo zone is due to the solubility of calcium carbonate in an acidic environment, even in dilute acid released from the colony (Hwanhlem et al. 2011). Variations of clear zones size on MRS-$CaCO_3$ agar reflect the amount of acid released from the colony (Phonyiam et al. 2008). The larger clear zone indicates the higher acid production (Than et al. 2022).

Cellulolytic LAB Screening

Cellulolytic bacteria are capable of producing cellulase and hydrolyzing cellulose into a simpler product, namely glucose. These microorganisms can degrade cellulose because they release enzymes with diverse specifications that work together. This enzyme will hydrolyze the bond (1,4)-ß-D-glucose in cellulose (Saratale et al. 2012). The data presented in Table 1 show that all isolates of lactic acid bacteria produce a clear zone indicating presence cellulolytic activity on CMC agar. Therefore, the test results are strong evidence that cellulase is produced to degrade cellulose (Lynd et al. 2002). In this study, a clear zone around the colony was observed after staining with Congo Red which then indicated CMC hydrolysis as a result of cellulase production and this was reported by Abdelnasser and Ahmed (2007). The hydrolysis product is a simple monosaccharide sugar and no complex bonds with Congo Red occur. According to Anand et al. (2009), Congo Red will bind specifically to polysaccharides that have β-1,4 glycosidic bonds, in this study the polysaccharides contained in the test medium is CMC. After staining, the media was then rinsed with 1M NaCl so that the clear zone can be seen properly. Among all isolates, F4 showed the largest clear zone indicating stronger cellulolytic activity. Narasimha et al. (2005) stated that cellulolytic activity continues to increase because bacteria are able to grow and reproduce where in the growth phase the bacteria produce primary metabolites in the form of cellulase enzymes so as to form a clear zone.

Cellulase Enzyme Activity

Based on the research, it was found that cellulase activity in 4 bacterial isolates ranged from 13.13-20.67U/mL. The cellulase activities of isolates F4, F6, F9 and F11 were 17.69, 20.67, 14.72 and 13.13U/mL, respectively. The best result was obtained by isolate F6. Cellulase activity is in line with the level of reducing sugar produced, if the activity of cellulase enzyme is high, the reducing sugar produced will also high. Differences in enzyme activity in LAB isolates could be caused by differences in the bacterial strains used. Bacteria with different strains produce different enzyme activities. This is in accordance with the opinion of Rietl et al. (2016) who stated that differences in microbial communities can produce various enzyme patterns which result in different enzyme activities between microbes. Some microbes can produce a high enzyme activity, but other enzymes have low activity (Yi et al. 2019). Therefore, the species of bacteria used can affect the activity of the enzymes produced. The cellulase enzyme activity obtained in this research was higher than that found by Akintola et al. (2019) who obtained cellulase enzyme activity of 4.38U/mL in *Enterobacter cloacae* IP8 bacteria using CMC as a carbon source.

Characterization of Lactic Acid Bacteria Isolates

Gram staining is a technique that can distinct bacteria into gram-positive and gram-negative bacteria (Yanestria et al. 2019). Gram positive bacteria will retain a crystal purple color and will therefore appear dark purple under a microscope. Gram-negative bacteria will lose their violet crystalline dye after washing with alcohol, and when given a dye equivalent to fuchsin or safranin water, it will appear red. The color difference that occurs is a result of the chemical structure of the different cell walls. Gram-negative bacteria have thinner peptidoglycan than gram-positive bacteria (Somani et al. 2023). Gram-positive bacteria exhibit a layer of peptidoglycan strands that can reach sizes between 30 and 100nm or even thicker, whereas gram-negative bacteria have a layer of only a few nanometers (Rohde et al. 2018). Based on observations,

Table 1: Clear zones of 4 LABs at CMC Media

Isolates	Diameter of cellulase clear zone (mm)
F4	21.5
F6	15.5
F9	15.0
F11	18.7

Table 2: Characteristics of LAB isolates

Characteristics	Isolate			
	F4	F6	F9	F11
Grams	+	+	+	+
Aerobic/anaerobic	aerobic	aerobic	aerobic	aerobic
TSIA	K/K	K/K	K/K	M/M
Gas	-	-	-	-
H2S _ _	-	-	-	-
Catalase	-	-	-	-
Oxidase	-	-	-	-
Mortality	-	-	-	-
Indole	-	-	-	-
Glucose	-	-	+	-
VP	+	+	+	-
arabinose	-	-	+	-
Nitrate	-	-	-	-
Maltose	-	-	-	-
Melibiose	-	-	-	-
Strains	*Lactobacillus* sp.1	*Lactobacillus* sp.1	*Lactobacillus* sp.2	*Lactobacillus* sp.3

the bacterial isolates F4, F6, F9 and F11 were gram-positive bacteria and aerobic. Aerobic bacteria are bacteria that require oxygen for growth.

sizes between 30 and 100nm or even thicker, whereas produced the catalase enzyme (Adebiyi et al. 2020). The catalase test was performed to find out whether the bacterial isolates produced the catalase enzyme (Adebiyi et al. 2020). Another study by Wang et al (2016) who isolated and identified LAB isolated from Traditional Dairy Products, reported that the isolated LAB also did not produce the catalase enzyme. This was proven by the absence of air bubbles in all isolates when H_2O_2 was dripped.

The TSIA (Triple Sugar-Iron Agar) test was performed to see the capability of bacteria to ferment glucose, lactose and produce acid. This study showed that the top and bottom were red (M/M) in F11 isolate indicated no carbohydrate fermentation while F4, F6 and F9 isolates showed yellow top and bottom (K/K), which indicated that these isolates fermented all carbohydrates (Gelgel et al. 2023). Furthermore, the formation of H_2S was not seen in all isolates indicating that these isolates were unable to decompose sulfur-containing amino acids.

The VP test (Voges-Proskauer) is a test used to discover acetonin in bacterial cultures (Poonam et al. 2022). The red color indicates a positive result, while the yellow color indicates brown or colorless is a negative result. The results showed a positive reaction on isolates F4, F6 and F9 while a negative reaction on isolate F11. Positive results indicate that the glucose fermentation product can form (acetoin) acetyl methyl carbinol while negative results indicate the organism does not form non-acid end products or neutral.

Oxidase test on all isolates showed negative results. This negative result can be seen from the absence of a blue color change when given the reagent (Yaqoob et al. 2022). The p-aminodemethylaniline oxalate reagent acts as an electron donor and will be oxidized to a blue compound if oxidase and free oxygen are present.

Indole testing is used to discover the presence of tryptophanase enzymes in bacteria that can hydrolyze the amino acid tryptophan to indole and pyruvic acid. The amino acid tryptophan is an amino acid that is commonly found in proteins, so that this amino acid can easily be used by microorganisms as a source of energy (Lay 1994). Based on the results of the study, it was found that all isolates showed a negative reaction on the indole test which indicated that all isolates did not have the tryptophanase enzyme.

sizes between 30 and 100nm or even thicker, whereas bacteria to carry out surface dispersal and the catalase test was performed to find out whether the bacterial isolates produced the catalase enzyme (Adebiyi et al. 2020).2022). Motile bacteria are characterized by spreading growth in the area around the ose puncture, while non-motile bacteria are characterized by the absence of the spread of bacteria in the area around the ose puncture. The results showed that all bacterial isolates were negative in the motility test.

Starch hydrolysis test (arabinose, melibiose, maltose) showed negative results on isolates F4, F6 and F11 while isolate F9 showed a positive reaction on arabinose. The difference in the use of starch can be seen from the color changes that occur in the media, if there is no enzyme that hydrolyzes starch. Iodine will make the color black if the starch is hydrolyzed it will look clear. A zone formed around the colony will indicate a positive result.

Test for the reduction of nitrate (NO_3) to nitrite (NO_2), after incubating organisms that reduce nitrate to nitrite will appear to produce a red color after adding reducing agent A which contains sulfinilat and alfanaphthylamine. If there is no change in color, the bacteria may have reductases that can reduce nitrites to ammonia or molecular nitrogen. All isolates showed negative reactions to the nitrate test.

Based on observations of colony morphology, cell morphology and physiological tests, namely the TSIA test, motility test, indole, nitrate, H_2S and gas formation for isolates F4, F6, F9 and F11 had the same characteristics as bacteria from the genus *Lactobacillus*, where isolates F4 and F6 were categorized as *Lactobacillus* sp.1, isolate F9 was marked as *Lactobacillus* sp.2 and isolate F11 was characterized as *Lactobacillus* sp.3.

Conclusion

In this study, 4 isolates of lactic acid bacteria were found to produce cellulase enzymes. Isolate F6 produced the highest cellulase enzyme, namely 20.67U/mL. All isolates found were gram positive and aerobic bacteria. Based on the characterization of the bacteria, isolates F4 and F6 were categorized as *Lactobacillus* sp.1, isolate F9 was marked as *Lactobacillus* sp.2 and isolate F11 was characterized as *Lactobacillus* sp.3.

Acknowledgment

This study is supported by funding from the PMDSU Grant of the Ministry of Research, Technology and Higher Education of the Republic of Indonesia for 2022 based on the “Decision of the Authorized Budget User Directorate of Research, Technology and Community Service with contract No: 086/E5/PG.02.00. PT/2022, Fiscal Year 2022.

Conflict of Interest

The authors have declared no conflict of interest.

Author's Contribution

Mirnawati, Yetti Marlida, Yose Rizal and Nurmiati developed the script concept. Anifah Srifani conducted experiments, analyzed data and wrote a script.

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