

Screening Of Phytase - Producing Bacteria As Probiotic Candidates Of Poultry

Muh. Aidil Fitriyan Fadjar Suryadi, Anwar Rosyidi, and Muhamad Ali*

Laboratory of Microbiology and Biotechnology, Faculty of Animal Sciences,
University of Mataram Jl. Majapahit No. 62 Mataram, Lombok, Indonesia,
m_ali@unam.ac.id

Abstract. This research aimed at screening of phytase-producing bacteria previously isolated from the gastrointestinal tract of poultry as probiotic candidates. Two screening indicators were used; (1) the capacity to produce phytase, and (2) the ability to grow in sugar media. This research was conducted at laboratory of Microbiology and Biotechnology, Faculty of Animal Science, University of Mataram for about 4 months since January to April 2019. Initially, bacteria were subculture in LB broth and on *agar* through serial dilutions to get a pure colony. Thereafter, the pure isolates were grown on sodium phytate-containing *agar*. The results shows that 4 bacteria had a capacity to produce phytase indicated by the formation of clearance zone on the sodium phytate-containing *agar*; three of them appeared to be member of lactic acid bacteria (LAB) indicated by their ability to ferment glucose. Since LAB members are generally regarded as safe (GRAS) microorganism, the results may suggest that the three bacterial isolates are potential probiont for poultry, although in vivo study using live poultry should be further investigated to get more comprehensive results.

Keywords: Bacteria; probiotic; poultry; phytase

1. Introduction

Rice bran is recidu of milling rice or grain into rice which has a by-product in the form of rice bran and husk. Rice bran is the results obtained between rice and the skin. Rice bran is a by-product of rice milling that cannot be stored for long periods. Rice bran has a protein content of around 12-14%, fat 7-9%, ash 9-12% and crude fiber 6-27% containing raw material for animal feed. In addition, rice bran has around 1600-1900 kcal/kg [1-2].

Rice bran has a relatively high nutrient content, and is used as a source of energy in animal feed. besides having a relatively high nutrient content, it also has abundant production and does not compete with humans, which will be able to streamline the use of imported raw materials of excessive feed [4]. Rice bran has the main disadvantage of having high enough crude fiber. In addition, bran contains low amino acids and contains phytic acid which binds minerals and proteins proteins to rice bran which can be difficult to digest by poultry digestive enzymes [5].

Phytic acid is a compound secondary which is used as a primary storage place for phosphate found in plant grains [5]. Besides containing phosphate, phytic acid also binds various types of minerals such as calcium, magnesium, and copper which can cause digestive mineral disruption. In addition to being able to bind minerals, phytic acid can bind to proteins so as to reduce the digestibility

of feed protein [6].

The existence of phytic acid is one of the obstacles for using rice bran in large quantities in the preparation of rations for monogastric animals, because there are phytic acid compounds. So, it is necessary to find a solution to break the bond of phytic acid, so that the nutrient and digestibility of rice bran increases.

One of the methods used to hydrolyze phytic acid content in rice bran is using phytase enzymes (indigenous or exogenous) by fermentation. Fermentation technology using microorganisms, one of the right ways to break down phytic acid content in rice bran. The basic principle of this technology is the breakdown of phytic acid by phytase enzymes produced by microorganisms that have been tested for their abilities [7].

The use of these technologies can reduce and even negate the impact of phytic acid without reducing livestock productivity and feeding efficiency [8]. Kies et al., [7], states that the feed added by phytase-producing enzymes is able to increase the absorption of 2 g / kg of phosphorus content.

The group of microorganisms that will be used and developed for this purpose is probiotics [9]. Probiotic is a product that contains non-pathogenic living microbes given to livestock with the aim of increasing the rate of livestock growth, efficiency of feed conversion and maintaining livestock health [10].

Various types of microorganisms used as probiotics are isolated from the intestinal contents of digestion, mouth, and animal manure. At present, microorganisms that are widely used as probiotics are strains of *Lactobacillus*, *Bifidobacterium*, *Bacillus* spp, *Streptococcus*, and *Saccharomyces cereviceae*. These microorganisms must be non-pathogenic, gram- positive, specific strains, anti-*E. coli*, resistant to bile, live, attached to the intestinal mucosa, and contain at least 30×10^9 CFU/g [11-12].

The ability of bacteria to produce phytase enzymes can increase the efficiency of the use of rice bran which contains phytic acid. Therefore researchers conducted research aimed at getting the bacteria that produce phytase enzymes to be used as probiotics in poultry.

2. Material and Methods

Preparation of Media LB (*Lysogeny Broth*) Agar

One-hundred millilitres LB Agar consisted of 1% *Trytone Powder*, 1% NaCl, 0.5 % *Yeast Extract*, 1.5 % Agar and 100 ml aquades. Then, mixture using *Hot Plat Stirrer* until homogen. The medium was homogen adjusted to be 7.2 with 0.1 M Sodium hydroxide (NaOH) or Hydrochloric acids (HCl). Then the mixture was autoclaved and ready to used.

Preparation Media LB (*Lysogeny Broth*)

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Isolation of Bacteries Phytase

The scrinning of bacteries done by first step was isolated bacteries from gastro intestinum of poultries. Then, test phytase, caracteritation, and biochemic. The isolation done with serial dulation (Figure 1) of bacteries was got from gastro intestinum. The solution of bacteries took 100 μ l and dulated on aquades 900 μ l with 3 dulation (10^{-1} , 10^{-2} , and 10^{-3}). Every dulation took 100 μ l which was growthed on LB Agar during 24 – 48 hours at incubator shaker using *spread plate* method.

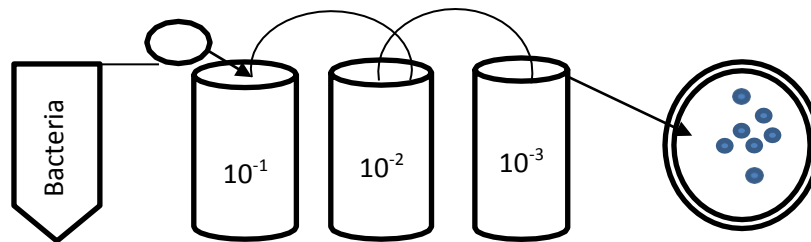


Figure 1. The Process Isolation of Bacteria

Bacteria has grown on media LB, then characterized to know morphology and difference of every colonies bacteria. Bacteria has morphology colony different spread and cultured in media liquid LB.

Biochemic Test (Phytase, Gram, Catalase, and Media Sugar Water) Phytase Test

The single colony of bacteria tested phytase to know ability of bacteria to produce enzyme phytase. Phytase test using media selection consisted of 1 g glucosa, 0,25 g NH_4NO_3 , 0,025 g KCl, 0,025 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0,1 g $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, 0,2 g Sodium phytat, 0,75 g Agar and 50 ml aquades (pH 5,5). The all ingredients of media heated up using *magnetic stirrer*. And then sterilization in autoclaved during 15 minutes at 121 °C with 15 lbs. Media was sterilization pour 15-20 ml in the petridisk, and then took 0,005 ml every isolate and *B. amyloliquefaciens* (control positive) with OD_{600} 0,2 and drop it in medium phytat, incubated in incubator during 24 - 48 hours at °C. After that, characterization which bacteria can cut content of phytat in media and measuring size of clearing zone surrounded growth of colony bacteria.

Gram Test (Coloration)

Coloration used Hucker method for bacteria cell purpose to know kind of cell bacteria using crystal violet, lugol, foxin and foxin.

Catalase Test

Catalase Test using 3 % hydrogen peroxide (H_2O_2). The marker of bacteria positive catalase has bubble gas surround of colony bacteria if there was not bubble gas in surround of colony bacteria that is negative catalase.

Sugar Water Test

Sugar water test purpose to know ability of bacteria growth in simple media using 20 g sugar on 100 ml aquades. The media sugar water test sterilized using autoclave at 121 °C on 15 lbs. after that, 5 μl isolate bacteria put in media sugar and incubated for 24 hours. The marker of bacteria that can growth on media sugar water was change clear media to be murky.

3. RESULTS AND DISCUSSION

The content of phytat acid in feed of poultry such as soybean and rice bran that was problem to maximal absorption protein and mineral phosphorus for poultry, one of solution to solving the problem using phytase enzyme that can cutting phytat held phosphorus [13]. For resulting phytase enzyme done using probiotic which has ability made phytase enzyme.

Isolation Of Producing-Phytase Bacteria

This study found 4 types of bacteria that can be used as phytase enzymes. The morphological forms of the four types of colonies are (1) bacteria colony A has a medium size, convex colony growth, there is a core point in the middle, jagged edge and white, (2) bacteria colony B has a large size, rounded, intact white and clear, (3) C bacteria colonies are round, small, convex, clear, white

color, and (4) bacteria D has oval, small, and clear colony. Figure 3 and Table 1 showed the results and bacterial growth in dilutions 10^{-3} .

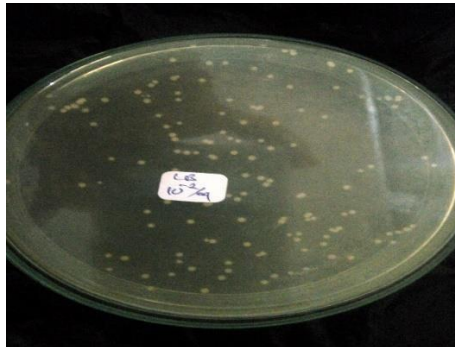


Figure 3. Some Colonies of Bacteria

The four types of bacteria colonies were purified through serial dilution and then grown on a liquid LB media that was incubated for 24 - 48 hours in a incubator shaker. Figure 4 show the results culture one of the bacteria that was successfully isolated. The Figure 4 resulted of the purification process of one of the bacterial colonies to obtain a single colony. Bacterial growth was marked by turbidity of liquid LB media which initially looks clear like the figure 4. Bacteria that grew in liquid LB media were then grown on solid LB media for 24 hours at 37 ° C in incubator shaker. Purification of the bacteria was more repeated until getting a single uniform colony as shown in Figure 5 and Table 1.



Figure 4. Culture one of bacteria isolate



Figure 5. The Single Colony One of Bacteria Biochemical Test

Scrinning Of Producing-Phytase Bacteries

This phytase-producing bacterial test was tested on solid LB media containing sodium phytate with a concentration 0.4% and as a control positive bacterium *Bacillus amyloliquefaciens* subsp. *Plantarum* (K). After the growth of several bacterial isolates in the agar media containing sodium phytate obtained the results of bacteria A, B, C, and D have the activity of breaking down phytic acid as indicated by the presence of a clearing zone on the culture media containing sodium phytate. Figure 6 and Table 1 showed the ability of bacteria to break down sodium phytate characterized by a clearing zone around the growing of colony.

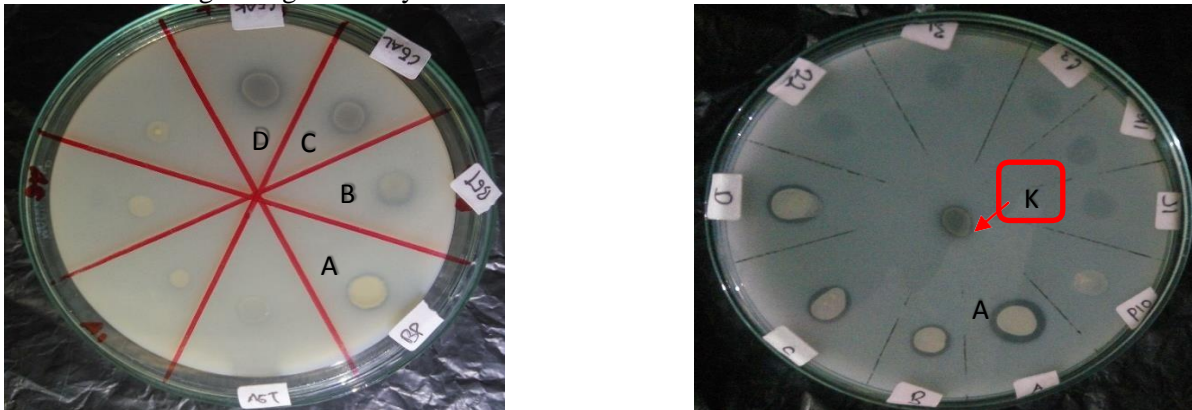


Figure 6. Result of phytase test bacterias (A, B, C, and D) in media LB

sodium phytate

This study accordance with what has been done by Kusdianto [14], who managed to isolate phytase-producing bacterial enzymes from the soil tested on selective media using sodium phytate. The presence of a clearing zone around the growth of a growing bacterial colony proves the bacteria as able to produce phytase.

This research has also been carried out by Amin and Liliyanti [15], who have succeeded in isolating and characterizing phytase-producing bacterial enzymes from fish intestines. The some of bacterias are tested on selective media and produce clearing zones around the growth of bacterial colonies and good isolates characterized by the highest clearing zone.

The ability of bacteria A (Bp), B (AGT), C (BGT), and D (Ct) to form a clearing zone indicates that these bacteria produce phytase enzymes that are able to hydrolyze the content of sodium phytate on solid LB medium containing sodium phytate. The bacteria D have the best ability to break down sodium which is characterized by the formation of the highest clearing zone based on the growth of the colony as accordance with the opinions of Amin and Liliyanti [14]. There is a clearing zone around of the growth of colonies caused by bacteria furthermore phytic acid hydrolysis into phosphorus (P) by extracellular phytase produced by bacteria A, B, C, and D which diffuses into the medium test.

The microorganism can result extracellular phytase which can hydrolyze content of phytate acid on feed being P that cause clearing zone around of growth colonies [16]. Probiotics produced are used as feed additives in foods to increase the availability of phosphate, by means of bacteria containing probiotics that have the ability to produce phytase enzymes will hydrolyze phytate reserves contained in feed ingredients such as rice bran.

These phytase producing enzymes can be used as feed additive for monogastric animals which are less able to produce phytase enzymes in their digestive tract. so, the adding up phytase enzymes by microorganisms that can produce phytase enzymes important to increase growth of poultry.

The Characteritaton of Bacteri Phytase Gram Test (Staining)

Gram staining was done to facilitate the process of identifying and knowing the morphology of

bacterial cells and to distinguish gram-positive and gram-negative bacteria (Rostinawati, 2008). Based on the results of gram staining that has been carried out microscopically with a magnification of 100 x obtained results of gram staining from bacteria A, B, C, and D as showed in Figure 7 and Table 1.

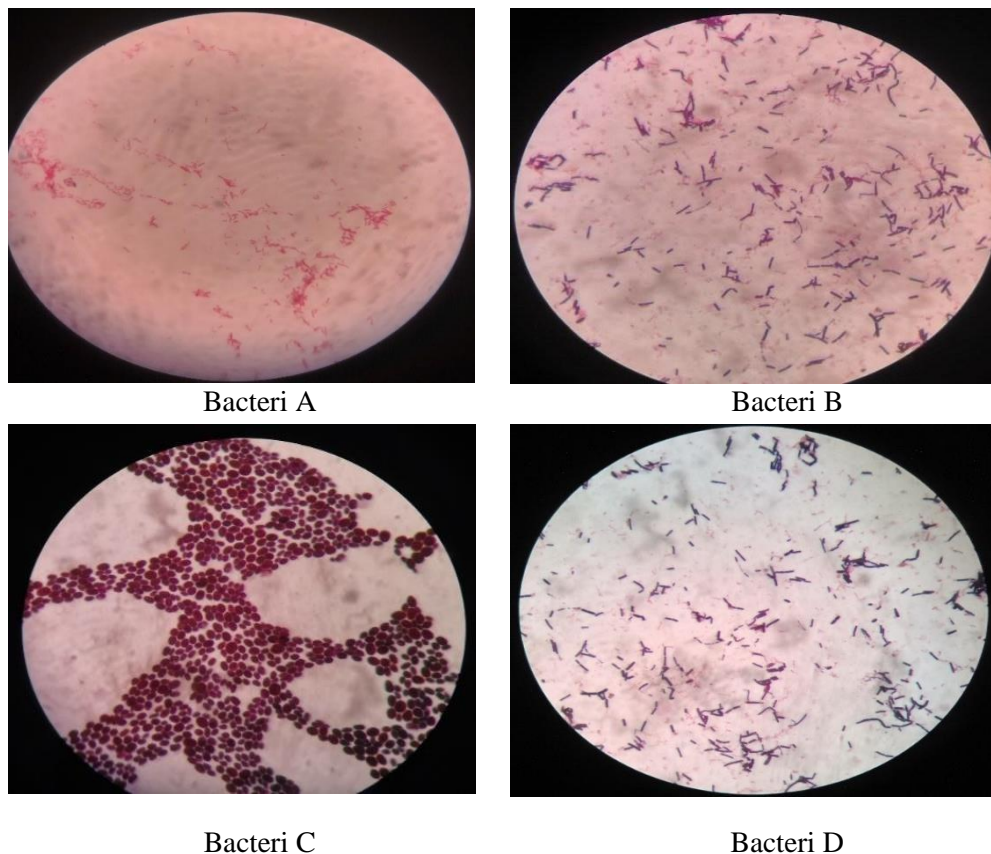


Figure 7. The result of staining gram

. Tabel 1. Morphology and Biochemic Analysis bacteries of digestivus poultry

No.	Code of Bacteries	Types coloni and size of bacteri	Color of colony	cells	Gram	Biochemic Test			Size of clearing zone	
						Fitase	Katalase	Sugar	Koloni (mm)	Zona bening (mm)
1	A (Bp)	Convex Middle	White	Bacilloc occuss	+	+	-	+	5	2
2	B (AGT)	Round Big	Clear	Bacill	+	+	-	-	5	1
3	C (BGT)	A little convex and round Small	Clear	Bacill	-	+	-	+	4	1
4	D (Ct)	Round and oval Small	Clear	Bacill	+	+	-	+	6	4

The figure 7 showed the cell of bacteria A is gram-positive *basillococcus*. The bacteria C is gram-negative *bacill*, while B and D bacteria are gram-positive *bacill*. Bacterial cells A, B, and D are purple indicating that they can bind to the color of violet crystals, whereas red or gram negative bacterial cells indicate that the bacteria cannot bind to the color of violet crystal and only colored by a solution of fuchsin (counter dye). Based on the results of morphological and biochemical analysis consisting of gram test, phytase test, catalase test and sugar media test on phytase-producing bacteria at Table 1.

The result of catalase test from bacteria A, B, C and D was got negative catalase results in Table 1, because they cannot produce air bubbles. The result of positive catalase test characterized by the formation of oxygen bubbles which showing results of the evolution produced by enzymes that convert hydrogen peroxide into air and oxygen [17].

Testing on Sugar Water Media

The ability of bacteria to grow in simple media such as sugar water will make it easier to make probiotics by using sugar water as a prebiotic. Special characteristics of lactic acid bacteria are able to grow at high levels of sugar, alcohol and salt, and also able to fermented monosaccharides and disaccharides [18].



Figure 8. The result of sugar water media

Based on the results of test of the ability bacteria A, B, C, and D to grow in pure sugar water getting results (Figure 8), only bacteria B is not able to grow in sugar water media.

CONCLUSION

The isolated bacteria (A, B, C and D) are able to produce phytase enzymes as evidenced by the presence of clearing zones around bacterial colonies that were grown on selective media. The bacteria B has the best ability of the four bacteria marked by the formation of the highest clearing zone around the growth of the colony. The existence of the clearing zone was caused by the phytic acid hydrolysis process by phytase enzymes, resulting in diffusion. Besides being able to produce phytase enzymes, it is also able to grow in sugar water media which will facilitate the making of probiotics.

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