Cultivation of Phytase-Producing Bacteria as Probiotic Candidate on Molasses and Tempe-Processing Waste

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Microbes that produce phytase enzymes are needed for livestock and fisheries. Animal feed treated with phytase will increase the availability of minerals, amino acids, and energy. For this reason, it is essential to growing phytase-producing bacteria on alternative media that are cheap and easy to obtain. This study aims to determine the ability of some bacteria that produce phytase to grow on alternative media such as molasses and tempeh waste. The indicators used were the growth rate of several phytase-producing bacteria within 3, 4, 5, and 6 hours of culture. The results showed that some bacteria were able to grow on alternative media of tempeh and molasses waste, although slower than growth on commercial media (LB). For this reason, tempeh and molasses waste can be used as alternative media to grow phytase-producing bacteria to produce cheap probiotics for livestock and fisheries.

1. Introduction

The animal industry consisting of livestock and fisheries must continue to be developed and intensified to meet the food needs of sustainable animal origin. Improving livestock quality must continue to be done by selecting, breeding, and improving nutrition, and avoiding the use of antibiotics in the livestock and fisheries industries because it can cause antibiotic resistance to pathogenic bacteria [1,2]. The use of probiotics helps improve the balance of the gut microbiome, suppress pathogens, improve gastrointestinal function, improve immunity, feed efficiency, increase productivity (meat, eggs, and milk), reduce morbidity and death, and host health [3,4,5,6,7].

Research must continue to find alternative antibiotic replacements, namely probiotics derived from single strain microbes or mixtures mixed in animal feed [8]. Increased digestibility of feed materials will increase livestock productivity and maximize growth [9]. The effectiveness of probiotic use depends mainly on the type of microbe used (single or mixed strain), viability, method of administration and dosage, environmental stress factors, and age and health status of animals [8].

About 80% of poultry animal feed comes from grains such as corn, soybeans, wheat, and various types of meals, which are sources of carbohydrates, fats, and proteins for livestock. In addition to containing nutrients needed by the body of animals, grains and meals contain anti-nutrient compounds for monogastric livestock, namely phytic acid. Phytic acid can bind to about 80% of the phosphorus in grains and inhibit enzymes including trypsin and amylase, thereby reducing protein digestibility in animals, inhibiting feed digestibility, and lowering the nutritional value of feed ingredients derived from agricultural crops [10,11,12]. As a result, it interferes with the utilization of feed and affects the
health and production of livestock through a mechanism of decreased nutrient intake [13]. So there needs to be a solution to break the phytic acid bond so that the digestibility of feed material increases in livestock [14].

Feed added bacteria producing phytase enzymes could increase the absorption of phosphorus content of feed, reduce the negative anti-nutrient influence of phytic acid and reduce feed costs [15]. Phytase bacteria that can grow in sugar water media [16], but not yet known the specific name will be tested for growth in the waste media treatment of tempeh and molasses to be used as a candidate for probiotics. The use of tempeh and molasses processing waste is an effort to use local materials that are abundant, cheap, and easy to obtain and has not been optimized for its use as a medium for bacterial growth in the process of making probiotics. Tempeh waste, in 100 g, contains 79 kcal, 0.04 g of fat, 0.047 g of protein, 4.06 g of carbohydrates, 94.55 g of water, and 0.88 g of ash [17], while molasses contain about 40-60% sucrose, glucose, and fructose in low concentrations. Both of these wastes are biodegradable, which is wastes or materials that can be destroyed by microorganisms [18].

2. Research Materials and Methods
2.1 Time and Place of Research
The research was conducted in March-June 2021 at the Microbiology and Biotechnology Laboratory of the Faculty of Animal Husbandry, Mataram University.

2.2 LB Media Creation (Lysogeny Broth) Liquid
The manufacture of liquid LB media is done by weighing tryptone powder (1%), NaCl (1%), yeast extract (0.5%) of the number of distilled water used volume then homogenized using a hot plate stirrer and measured pH (7.2) [19,20,21]. The media is inserted into a test tube of as much as 5 ml and then sterilized using an autoclave.

2.3 LB Media Manufacturing (Lysogeny Broth) Solid
The manufacture of solid LB media is done by weighing tryptone powder (1%), NaCl (1%), yeast extract (0.5%), and agar (1%) of the number of distilled water used volume then homogenized using a hot plate stirrer and measured pH (7.2) [19,20,21]. After that, the LB media is sterilized using autoclave for ± 15 minutes then poured into a petri dish (25 ml) that has been sterilized.

2.4 Manufacture of Glucose Tempeh Wastewater Media
Soy boiled water is first filtered. Then added 2% glucose in 100 ml of soy-boiled water then homogenized using a hot plate stirrer. pH measured if the acid then added 2N NaOH to pH 7.2. After that, 5 ml of media is inserted into the test tube and sterilized using an autoclave for ± 15 minutes.

2.5 Manufacture of Waste Water Media Tempe Molasses
The manufacture of tempeh waste and molasses is done by weighing Urea 8.5 mg/ml, Monopotassium phosphate 3.1 mg/ml (KH2PO4), Magnesium sulfate 1.71 mg/ml (MgSO4), soy decoction wastewater (5%), and molasses (10%) that have been filtered from the amount of distilled water volume used as solvents. The media is homogenized using a hot plate stirrer and measured pH (7.2). The media is inserted into a test tube of as much as 5 ml and then sterilized using an autoclave.

2.6 Creation of phytase Media
The medium used in conducting the phytase test is 1.5 g glucose, 0.375 g NH₄NO₃, 0.0375 g MgSO₄ 7H₂O, 0.0375 g KCl, 0.15 g CaCl₂:H₂O, 0.3 g Sodium Fitate and 1.125 g Agar inserted into the Erlenmeyer containing 75 ml of distilled water (pH 5.5). After that, the media is heated while homogenized using a hot plate stirrer. Sterilization is performed by autoclave for 15 minutes at a temperature of 121 °C with a pressure of 15 lbs. Once the media is sterilized, the media is poured on a sterile petri dish and awaited freezing.

2.7 Rejuvenation of Probiotic Candidate Bacteria Isolates
Five bacterial isolates with isolate codes A, AGT(B), and BGT(C), CET code (D), and Bacillus amyloliquefaciens subsp plantarum (P10) isolates previously isolated by Suryadi [16] were rejuvenated in liquid LB media with a culture time of 24 hours at a speed of 120 rpm at 37 °C using shakers. After that, a streak is done on solid LB media using the quadrant method and then incubated for 24 hours with a temperature of 37 °C.
2.8 Characteristics of Probiotic Candidate Bacteria Isolates

Bacterial isolates that have been cultured are characterized again by observing the morphological form of bacteria macroscopically (morphologically) and microscopic (Gram painting method) [22].

2.9 Phytase Test

A Blank Disc is affixed to the phytase media and dripped as much as 25 μl isolate bacteria (OD600 0.2) on a blank disc. Bakteri *B. amyloliquefaciens* have been tested for phytase as a positive control. Then wait until dry and incubate in an incubator for 24 hours at a temperature of 37 °C. After incapacitation, measurements are made of bacteria that are able to break down the phytic sodium content by measuring the length of isolated growth and the size of the clear zone formed around the growth of the colony.

2.10 Starter Creation

Starter manufacturing is done by taking a single colony corresponding to Suryadi [16] from a solid LB to be injected on a liquid LB and incubated on a shaker incubator for 24 hours (O/N) at a speed of 120 rpm at a temperature of 37 °C.

2.11 Test bacterial growth

Each test tube containing each liquid medium, tempeh + glucose waste media, and tempeh waste media + molasses is added 50 μl starter and incubated at a temperature of 37 °C using a shaker at a speed of 120 rpm. Measurements of bacterial growth are carried out after culture time of 3, 4, 5, and 6 hours at each isolate by taking 1 ml of culture plus 1 ml of distilled water then homogenized and measured turbidity using a UV-Vis (Ultra Violet-Visible) spectrophotometer with a wavelength of 600 nm.

2.12 Measurement of Bacterial Biomass

Cultures that had been incubated on shakers during *overnights* at 37 °C were transferred to the Effendorf tube for centrifugation at 12000 rpm for 2 minutes. Then the pellets are stored, and the supernatant is discarded. Eppendorf tubes containing bacterial pellets are weighed using analytical scales. The weight of bacterial biomass is tube mass difference having pellets with the mass of an empty tube.

3. Results and Discussions

3.1 Characterization of bacteria

The results of bacterial isolate rejuvenation with isolate codes A, AGT(B), and BGT (C) and CET(D), and P10 (Table 1) were observed macroscopically [23]. Morphological characterization is helpful for knowing the shape of the colony, the color of the colony, the edge of the colony, and the elevation of the cultured bacterial colony. Macroscopic differences can be caused due to differences in the growing media used. This macroscopic difference can be used as an initial benchmark in the process of purification of bacteria. Based on the results of morphological observations of colonies obtained a round shape (100%). The colors of the growing colony are white (60%) and cream (40%). The edges of the colony are flat (80%) and jagged (20%). The elevation is in the form of umbonate (20%) and convex (80%).

**Table 1. Morphological Observations of Bacterial Colonies**

| No. | Isolated Bacteria | Colony Shape | Color of Colony | Edge of the Colony | Colony Elevation |
|-----|-------------------|--------------|-----------------|-------------------|-----------------|
| 1   | A                 | Round        | White           | Flat              | Umbonate        |
| 2   | B                 | Round        | White           | Flat              | Low-convex      |
| 3   | C                 | Round        | Beige           | Flat              | Low-convex      |
| 4   | D                 | Round        | White           | Flat              | Convex          |
| 5   | P10               | Round        | Beige           | Jagged            | Convex          |
After making morphological observations macroscopic, microscopic morphological observations are made by doing Gram staining. Gram staining aims to find out the group of bacteria. Based on Gram staining, the characteristics of bacteria are divided into two groups, namely Gram-positive bacteria and Gram-negative bacteria. Gram-positive will be purple, and Gram-negative bacteria will be red. In addition, gram painting is also done to find out the shape of bacterial cells [24,25,26,27]. Based on Table 2, the results of bacterial cell observations from Gram staining, obtained bacterial isolates A, B, C, D, and P10, including Gram-positive (100%) shaped basil cells (stems) (80%) and D isolates have a coccus cell shape (round) (20%).

Table 2. Results of Gram Coloring of Bacterial Isolates

| No. | Isolates | Cell shape | Types of Grams |
|-----|----------|------------|----------------|
| 1   | A        | Basil      | +              |
| 2   | B        | Basil      | +              |
| 3   | C        | Basil      | +              |
| 4   | D        | Coccus     | +              |
| 5   | P10      | Basil      | +              |

3.2 Verification of Phytase Enzyme-Producing Bacteria

The results of the phytase test use a medium containing sodium phytate and *B. amyloliquefaciens subsp. plantarum* as a positive control results in a clearing zone around a colony that grows on a medium (Figure 1). The indicates that isolates A, B, C, and D have activity in breaking down phytic acid.

![Figure 1](image1.png)

**Figure 1.** Results of bacterial phytase tests A, B, C, D, and Control (K) in the medium sodium phytate.

The results of this phase test showed that the bacteria D has a larger clearing zone (1.9 cm) than isolate A (d; 1.5 cm), isolate B (d; 1.6 cm), and isolate C (d; 1.6 cm). The difference in diameter in each isolate is due to differences in the ability of the isolate to whiten phytic acid into phosphorus. According to Selle et al. [28] that microorganisms that produce extracellular phytase are able to hydrolyze phytic acid into phosphorus so that the area around the colony looks clear. Phosphorus produced from the process of decomposing phytic acid is dissolved in the media so that turbidity in the selling of the colony is lost (Figure 1).

3.3 Growth of Phytase-Producing Bacteria in Various Mediums

This study uses *Lysogeny Broth* (LB) media as a commercial medium (control) which is media for growing bacteria because it has nutrients that are in accordance with the growing needs of bacteria. However, high prices and relying on imports become one of the obstacles and in obtaining commercial media materials.

This research tries to utilize waste that is available abundantly as an alternative medium for bacterial growth at affordable prices and easy to obtain, and has excellent nutritional content for bacterial collisions in the form of tempeh waste and sugar factory waste (Molasses). Observation of bacterial isolate growth is done by measuring the level of cell density (A, B, C, D, and P10) based on culture time of 3, 4, 5, and 6 hours on each bacterial isolate and media based on the turbidity of phytase enzyme-producing bacterial isolates on LB media, glucose tempeh waste media and molasses tempeh waste media using UV-Vis (Ultra Violet-Visible) spectrophotometers to see turbidity (Optical Density) which reads (OD600) through the resulting absorbance value [29].
Based on The Analysis of variance (ANOVA) (Table 3) shows that the growth of phytase enzyme-producing bacteria in LB media, glucose tempeh waste, and molasses tempeh waste with different culture times results in the growth of different bacteria in each medium due to the result of a calculation significance value smaller than 0.05 (P<0.05) which means it has a noticeable influence (H0 is rejected). This is because the growth rate of phytase enzyme-producing bacterial isolates measured based on OD600 values in LB media is faster to grow than in glucose tempeh waste media and molasses tempeh waste media.

There is a glucose tempeh waste medium and the molasses tempeh waste in the first 3 hours still indicates a phytase adaptation. Entering the culture time of 4 hours to 6 hours, the growth of bacterial isolate shows a significant increase and has entered an exponential phase. The 3-hour adaptation period includes a relatively short time for bacterial growth. This is in accordance with the opinion of Yuliana [30] that the short phase of bacterial adaptation due to the same medium at the time of refreshment causes a short time of adjustment to the new environment. By 3 to 4 hours, the growth curve of phytase-producing bacteria has entered an exponential phase that indicates constant growth in bacterial cells. This is supported by Rainy [31] opinion in Sharah et al. [32] that within 3 to 4 hours, there is a logarithmic phase characterized by significant growth of its cells by dividing at a constant rate, the period is doubled at the same rate, regular metabolic activity, and a balanced state of growth.

Bacterial isolates A, B, C, D, and P10 have been shown to produce phytase enzymes and are able to grow on molasses tempeh waste media. This will facilitate the manufacture of probiotics. The combination of isolates capable of producing phytase enzymes with growing media can reduce phytic levels and can support the manufacture of probiotics as complementary feed ingredients that will be given to livestock.

**Table 3.** Results of measurement of OD (Optical Density) isolates bacteria producing phytase enzymes

| Media Perlakuan | Isolated Bacteria | Culture Time (hours) | (± STDV) | Total Treatment |
|-----------------|-------------------|----------------------|----------|----------------|
|                 |                   | 3                   | 4       | 5              | 6               |                      |
| Lysogeny Broth (LB) |                   |                      |          |                |                |                      |
| A                | 0.365 ± 0.008     | 0.457 ± 0.014       | 0.555 ± 0.023 | 0.644 ± 0.034 | 0.50975 ± 0.1088h |
| B                | 0.398 ± 0.009     | 0.535 ± 0.036       | 0.564 ± 0.032 | 0.604 ± 0.022 | 0.52533 ± 0.0840b |
| C                | 0.21 ± 0.007      | 0.443 ± 0.044       | 0.494 ± 0.055 | 0.522 ± 0.062 | 0.41700 ± 0.1346g |
| D                | 0.431 ± 0.041     | 0.564 ± 0.036       | 0.647 ± 0.064 | 0.713 ± 0.065 | 0.58875 ± 0.1187i |
| P10              | 0.238 ± 0.007     | 0.442 ± 0.022       | 0.500 ± 0.047 | 0.534 ± 0.054 | 0.43000 ± 0.1212g |
| Glucose Tempeh Waste |                   |                      |          |                |                |                      |
| A                | 0.086 ± 0.051     | 0.258 ± 0.058       | 0.274 ± 0.012 | 0.313 ± 0.010 | 0.23258 ± 0.9695α |
| B                | 0.171 ± 0.056     | 0.269 ± 0.014       | 0.284 ± 0.010 | 0.328 ± 0.015 | 0.26292 ± 0.6509dc |
| C                | 0.047 ± 0.025     | 0.164 ± 0.017       | 0.197 ± 0.003 | 0.244 ± 0.009 | 0.16267 ± 0.7715sa |
| D                | 0.231 ± 0.146     | 0.374 ± 0.088       | 0.423 ± 0.059 | 0.447 ± 0.053 | 0.36892 ± 0.1189f |
| P10              | 0.049 ± 0.024     | 0.173 ± 0.017       | 0.205 ± 0.018 | 0.233 ± 0.004 | 0.16500 ± 0.7501a |
| Molasses Tempeh Waste |                   |                      |          |                |                |                      |
| A                | 0.137 ± 0.04      | 0.272 ± 0.063       | 0.305 ± 0.016 | 0.35 ± 0.026  | 0.26600 ± 0.8814azc |
| B                | 0.154 ± 0.016     | 0.227 ± 0.055       | 0.332 ± 0.029 | 0.365 ± 0.020 | 0.26975 ± 0.921v |
| C                | 0.05 ± 0.006      | 0.176 ± 0.009       | 0.246 ± 0.035 | 0.300 ± 0.025 | 0.19300 ± 0.0994ab |
| D                | 0.152 ± 0.048     | 0.315 ± 0.080       | 0.327 ± 0.031 | 0.376 ± 0.019 | 0.29250 ± 0.9755e |
| P10              | 0.067 ± 0.012     | 0.231 ± 0.019       | 0.265 ± 0.027 | 0.338 ± 0.045 | 0.22533 ± 0.1063bc |

Description: Different superscripts in the same column show a noticeable difference (P<0.05)
3.4 Measurement of Biomass Isolates of Bacteria Producing Phytate Enzymes in Various Media

Based on the results of the Analysis of variance (ANOVA) showed that there was no noticeable difference (P>0.05) in the weight of the pellets isolating the enzyme phytase enzyme in LB media, glucose tempeh waste, and waste tempeh molasses (Table 4).

Table 4. Biomass Production of Phytase enzyme-producing bacteria in various media

| No | Isolat | LB (g)       | Glucose Tempeh Waste (g) | Molasses Tempeh Waste (g) |
|----|--------|--------------|--------------------------|---------------------------|
| 1  | A      | 0.05 ± 0.02  | 0.04 ± 0.01              | 0.03 ± 0.02               |
| 2  | B      | 0.05 ± 0.03  | 0.04 ± 0.01              | 0.03 ± 0.01               |
| 3  | C      | 0.05 ± 0.02  | 0.05 ± 0              | 0.03 ± 0.01               |
| 4  | D      | 0.05 ± 0.01  | 0.05 ± 0.03              | 0.03 ± 0.01               |
| 5  | P10    | 0.03 ± 0.01  | 0.05 ± 0.02              | 0.04 ± 0.02               |

Table 4 shows that bacteria A, B, C, and D have the same average pellet weight on LB media which is 0.05 g/ml, and P10 bacteria of 0.03 g/ml. The average weight of insulated pellets A, B, C, D, and P10 in glucose tempeh waste media is almost the same in LB media, about 0.04 g/ml and 0.05 g/ml. In molasses waste media, isolates A, B, C, and D have an average pellet weight of 0.03 g/ml, while P10 bacterial isolates are 0.04 g/ml. Biomass growth will continue to increase to the maximum point over time and will decrease due to aging and bacterial death.

Based on the description above, the alternative media of glucose and molasses waste is suitable for use as a growing media for bacterial isolates producing phytase enzymes because of the content possessed by tempeh waste, namely proteins, fats, water, and several other minerals [33]. The nutrient content in sugar waste (molasses) is high enough to grow bacteria well. The presence of nutrients contained in each media will increases the ability to grow to isolate bacteria producing phytase enzymes.

4. Conclusion

Bacterial isolates A, B, C, D, and P10 producing phytase enzymes are able to grow well in alternative mediums of glucose tempeh waste and molasses characterized by clearing zones around colonies of bacterial isolates that are grown in media containing phytic acid.

5. References

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