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Development of Growth Media for Bacillus *amyloliquefaciens* subsp. *plantarum*, a Poultry Probiotic Candidate

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Abstract
Bacillus amiloliquefaciens subsp. *plantarum* is a poultry probiotic candidate due to its ability to produce three important digestive enzymes; protease, amylase, and cellulase. In its application, the bacterial isolate needs to be cultivated in mass scale, therefore requires a lot of growth medium. Thus, this study aimed at developing low-cost media to replace expensive commercial growth media for culturing the probiont. Three media (two media developed by using two local sources) and one commercial LB medium as the control were used to culture *B. amiloliquefaciens* subsp. *plantarum*. Growth was measured in terms of Optical Density (OD), and total harvested bacterial biomass (mg). The results indicated that there was no significant difference in OD of the probiont measured at the three media, which indicates that both local source-based media could support the growth of the probiont at the same rate as the commercial medium. Furthermore, total biomass of the probiont harvested from palm sugar medium was significantly higher (12.130 mg/mL), compared to either tofu wastewater (10.870 mg/mL), or LB medium (7.800 mg/mL). This result suggests that palm sugar medium was the most suitable medium for culturing *B. amyloliquefaciens* subsp. *plantarum* on a mass scale. However, the quantity and activity of enzyme synthesized by the bacterium cultured in the local source medium should be further investigated.

Keywords: *Bacillus amyloliquefaciens,* LB media, palm sugar, tofu wastewater, growth

INTRODUCTION

About 70-80% of total production cost in animal husbandry including poultry comes from feed. Poultry feed in Indonesia especially broilers and layers, relies heavily on imported proteinaceous feedstuffs including soybean meal and corns [1]. Recent data indicated that about 2.4 and 2.26 million tons of corn and soybean respectivelly were imported to Indonesia during 2016 to fulfill the requirements [2]. The reliance on the imported feedstuffs has resulted in large increases of total feed cost. In the recent years, the use of local feed ingredients such as local farmed corn and rice bran, were promoted to produce more sustainable meat production and to improve livestock production efficiency [3]. The local feed materials are not only available to the farmer with the cheaper prize but also can be continuously obtained. However, poorer nutritional value of the local components hampered its optimal application in poultry.

Improvements of digestibility of local feedstuff with supplementation of digestive enzyme-producing bacteria, commonly known as probionts, is considered to increase portion of the local feedstuff in ration. The use of probiotic can enhance the digestibility of local feedstuff for reducing feed cost. One of probiotic strains generally used as probiotics is *Bacillus amyloliquefaciens* subsp. *plantarum.* This bacterium is Gram-positive, bacilli of cell shape, and produces endospore in ellipse [4]. Wizna *et al* [5] reported that the bacterium can be used to ferment rice bran for broiler chicken feed.

According to Atlas [6], nutrient content in growth media determined growth rate of bacteria. Therefore, medium composition is the most important component in the bacteria cultivation. There are several commercial media which are available for cultivating bacteria including: *Lysogeny Borth* (LB)*, Natrient Agar* (NA)*, Eosin Methylene Blue Agar* (EMBA)*, Nutrient Broth* (NB)*,* and *Salmonella Shigella Agar* (SSA)*.* However, these growth media are very expensive, because they should be imported from other countries. As consequence, many researchers get difficulties in getting these growth media. Thus, alternative media based on local sources are urgently needed. Such media are required to support researchers conducting microbial studies or to supply industries performing mass microbial production.

This present study developed growth media using 2 local materials (either palm sugar or wastewater from tofu production). Palm sugar is considered to be rich in nutrient content, containing growth energy 368 kilocalori, calsium 75 mg, fosfor 35 mg, dan iron 3 mg [7]. In addition, palm sugar has vitamins such as vitamin A, vitamin B1 and vitamin C [8]. Meanwhile, wastewater from tofu production has high protein content protein 5,29%, fat 0,54%, and other important minerals [9]. Based on this nutrient content, it was hypothesized that palm sugar and the tofu wastewater could be used as media to support the growth of *B. amyloliquefaciens* subsp. *plantarum*.

MATERIALS and METHODS

Probiotic strain

This study used *B. amyloliquefaciens,* a poultry probiont which had capacity to produce three digestive enzymes (protease, amylase and cellulase). The probiont was one of poultry probiont collection in poultry Laboratory, Facultry of Animal Science, University of Mataram. It was recovered from glycerol stock according to Amin *et al* [10]. In brief, 10 ul of bacterium in the glycerol stock was inoculated in a 10-mL LB medium and incubated aerobically at 37°C for 48 h. Then the broth culture was streaked on LB agar, and incubated at the same temperature as above. After 48 h, a single separate colony was taken for gram staining, and catalase test to make sure that the bacterial strain was correct. Then, another single colony was picked and inoculated into a larger scale medium (200 mL) as starter culture, incubated at 37°C aerobically at shaker with 200 rpm for 3 days.

Preparation Palm sugar broth (PSB)

The PSB medium was prepared by weighing these components: palm sugar 20 g/L, MgSO4 0.38 g/L, K2HPO4 0.6 g/L, and NaCl 2 g/L. All these materials were added into one liter of distilled water (dH2O). The medium was placed on a hot plate stirrer to dissolve completely all the materials. Thereafter, pH of the medium was adjusted to 7.2 with 2 N sodium hydroxide (NaOH). Then, the medium was poured into 5 mL bottle and placed into an autoclave for sterilization.

Preparation of Tofu wastewater broth (WWB)

Fresh tofu wastewater was collected from a home industry of tofu. Then the wastewater was autoclaved to reduce contaminant and centrifuged at 8,000 rpm for 15 min. The obtained supernatant was collected and pH was adjusted to 7.2 by adding 2 N NaOH. Thereafter, glucose was added at concentration 0.2 g/L. The media was afterward sterilized in an autoclave.

Preparation of LB broth (LBB)

LBB medium was prepared by weighing these materials: peptone 10 g/L, yeast extract 5 g/L, and sodium chloride (NaCl) 5 g/L. These materials were mixed in distilled water (dH2O) and placed on the hot plate stirrer to dissolved the materials. Then pH was adjusted to 7.2 with 2 N NaOH. Then the medium was poured into a 5 mL bottle and sterilized in the autoclave.

Preparation of bacterial starter and cell harvesting

Cell harvestig and preparation of bacterial starter were performed according to Amin *et al* [11] with some modification. Briefly, a fresh culture of a pure *B. amyloliquefaciens* subsp. *plantarum* cultured in 200 mL LB broth was centrifuged at 3,500 x g for 15 min at 4°C using an Eppendorf Centrifuge. Supernatant was removed and pellet was washed by phosphate buffered saline (PBS). The mixture of bacterium and PBS was centrifuged again at the same condition as above. Supernatant was removed and pellet was resuspended in sterile PBS to a concentration of approximately OD_{600} : 0.4.

Inoculation and growth observation

Inoculation of the bacterium was conducted according to Amin [12] with some modifications. Each medium had 5mL volume with three replicates of each. The 5mL medium was inoculated with 50µl of microbial starter (OD_{600}:0.4). Since the bacterial starter was taken from the same dilution, it was assumed that each medium was inoculated with the same cell concentration of *B. amyloliquefaciens* subsp. *plantarum*. Thereafter, the growth of bacterium was monitored by measuring optical density (OD) at 600 nm of wave length, at several time intervals: 0 h, 3 h, 3 h, 4 h, 4.5 h, 5 h, 5.5 h, 6 h, and 6.5 h.

Data analysis

Growth of *B. amyloliquefaciens* subsp. *plantarum* in terms of OD_{600} values was compared using one-way analysis of variance (ANOVA) at $P<0.05$. Any differences detected among the treatments were continued with a Tukey post hoc analysis at $P<0.05$.

RESULTS

Colony and cell Morphology

Morphologically, an overnight culture of *B. amyloliquefaciens* MA228 colony appeared to be white, circular and opaque. Under a microscope, the cells were rod-shaped with rounded ends, and generally occurred in a single cell or in chains (Fig. 1a & 1b). In addition, the bacterium was a Gram-positive and able to produce catalase and oxidase. Summary of phenotypic characteristics of *B. amyloliquefaciens* subsp. *plantarum* was presented in Table 1.

Table 1. Characteristic of *B. amyloliquefaciens* MA228 cultured in agar media.

Characteristic of Bacteriaum	Observation Results
Colony Morphology	
Shape	Circular
Margin	Entire
Elevation	Flat
Size	Large
Texture	Rugose (wrinkled)
Color	White
Appearance	Dull
Pigmentation	Nonpigmented (white)
Optical property	Opaque
Cell Morphology	
Appearance	Bacillus
Arrangement	Mostly single
Biochemical characteristics	
Gram staining	Positive
Catalase	Positive
Oxidase	Negative

***The growth of B. amyloliquefaciens* subsp. *plantarum* in three different broth media (palm sugar, Tofu wastewater and LB).**

Growth of the bacterium in the three broth media was investigated by measuring optical density (OD) at 600 nm of wave length using a spectrophotometer. In general, the result showed that there was no significant difference of OD value among the three growth media, $p>0.05$. As shown in Fig. 2, the growth of *B. amyloliquefaciens* subsp. *plantarum* after 3 h incubation in the wastewater as fast as in the LB, but lower than in palm sugar. However, the growth of the bacterium after 3.5 h in the wastewater was slightly lower than the other two growth media, especially at 5 h, 5.5 h and 6 h of incubation time.

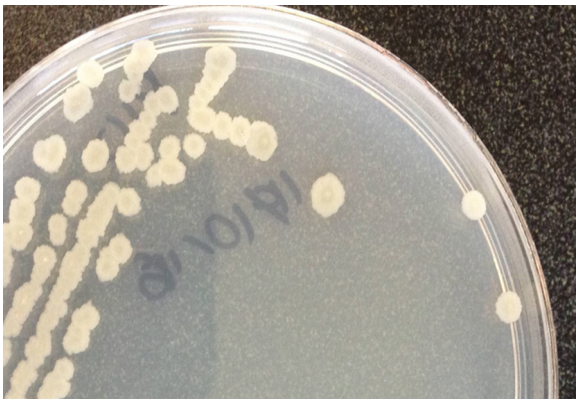


Figure 1. (a)

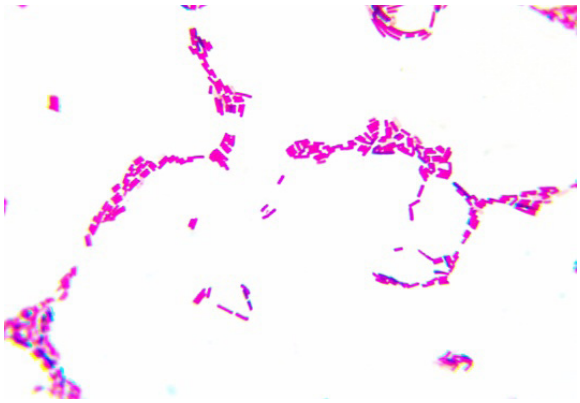


Figure 1. (b)

Figure 1. (a) morphology of *B. amyloliquefaciens* MA228 colony on LB agar. **(b)** cell morphology of *B. amyloliquefaciens* MA228 under microscope with 100x magnification.

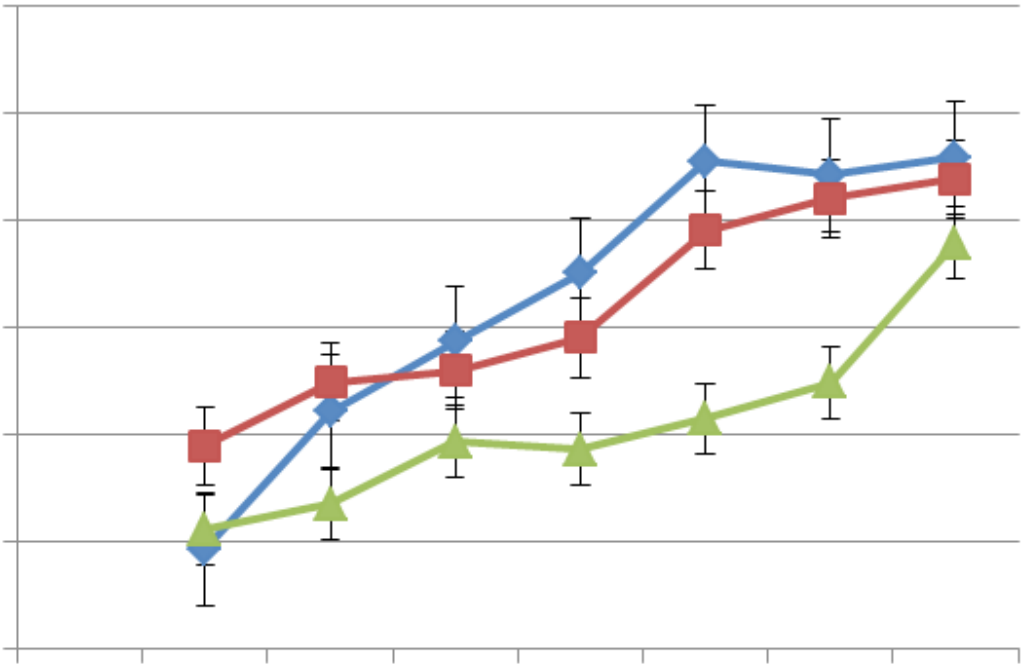


Figure 2. The growth of *B. amyloliquefaciens* MA228 at three different media presented in terms of OD_{600} value. Blue line is LB, red is palm sugar media and green is tofu wastewater media.

Table 2. Nutrient composition of growth media

Growth media	Components	Reference
LB	Casein enzymic hydrolysate, 10 g/L Sodium chloride, 10 g/L Yeast extract, 5 g/L	[19]
Palm sugar	Sucrose 71%, Glucose 3%, Fructose 3%, Nutrients, inulin and Antioxidants 22%	[7]
Tofu wastewater	Carbohydrate (2%), Calcium (0.1%) and Iron (0.1%)	[20]

Interestingly, the growth of the probiotic since 6 h cultivation in wastewater media was increased exponentially to reach the population density near the LB and palm sugar media. Thus, the final population density of the bacteria after 6.5 h cultivation in the two local media as high as in comercial media (LB).

Biomass growth of B. amyloliquefaciens* subsp. *plantarum

In terms of total generated biomass, the bacterial cells harvested after 48 h incubation period was significantly different among the three media, $P<0.05$. The highest amount of biomass was collected from bacterium cultivated in palm sugar media (12.130 mg/mL), followed by bacterium harvested from tofu wastewater medium (10.870 mg/mL), and the lowest was bacterium cultured in the commercial LB

medium (7.800 mg/mL), Figure 3.

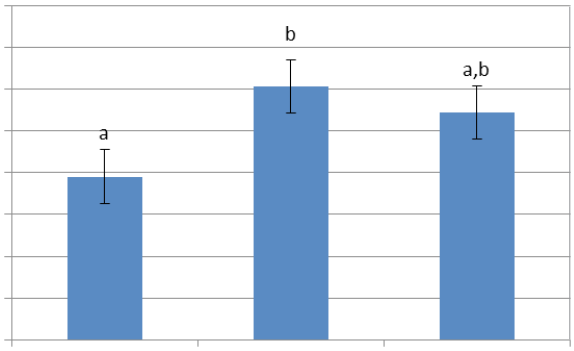


Figure 3. Total biomass of *B. amyloliquefaciens* MA228 harvested from three different media after being incubated aerobically for 6.5h at 37°C.

Comparing to another research result, wet weight of total biomass of *B. amyloliquefaciens* harvested from commercial medium (LB) as high as wet weight of total biomass of *Escherichia coli* cultured in the same media using conventional (shaking) technique [13]. Interestingly, the best result in the experiment was lower than the wet weight of total biomass of *B. amyloliquefaciens* subsp. *plantarum* harvested from palm-sugar based medium.

DISCUSSION

Commercial media for culturing bacteria are generally expensive and difficult to obtain especially in remote areas [13]. Thus, this study aimed at developing growth media based on local sources to replace commercial growth media. Two growth media developed from 2 local materials (either palm sugar and tofu wastewater) and a commercial growth medium (LB) were compared to grow a poultry probiotic candidate, *B. amyloliquefaciens* subsp. *plantarum*. The LB was used as a control because the medium has been widely used as the growth medium for bacteria including bacillus groups [14].

The result showed that there was no significant difference in the growth of the probiotic using the three media. Started from no significant different of OD value at 0 h, there was statistically no different in OD measured after 4.5 h incubation. However, a slight difference was started to be observed after 5 h incubation, where both LB and palm sugar media had higher growth of the probiont than tofu wastewater. The growth difference was continuously viewed until 6 h incubation. However, after 6 h, *B. amyloliquefaciens* subsp. *plantarum* showed sharp growth in tofu wastewater, and reach the same OD as the other two growth media (LB and palm sugar). This result may indicate that nutrient content in the tofu-wastewater was less digestible at the beginning (0-6 h incubation) than the other two growth media [15]. In addition, this result also suggests that the bacterium produce unknown extracellular products after 6 h which made nutrient content in the medium more digestible.

The other result indicated that total bacterial biomass of *B. amyloliquefaciens* subsp. *plantarum* harvested from palm sugar was significantly higher than total biomass collected from LB media and tofu wastewater. This result may suggest that palm sugar medium provides more nutrient contents required for growing *B. amyloliquefaciens* subsp. *plantarum* than either LB or tofu-wastewater media. The palm sugar is rich with carbohydrate (2%), calcium (0.1%) and iron (0.1%) [16]. Other source stated that palm sugar consists of sucrose 71%, glucose 3%, fructose 3%, nutrients, inulin, and antioxidants 22% (https://food.ndtv.com) which are required for growing genus bacillus. The better growth of *B. amyloliquefaciens* subsp. *plantarum* in the palm sugar medium might indicate that nutrient content in the growth medium could support the nutrient requirement of the probiotic strain Ganjar *et al* [17].

Furthermore, the higher total biomass obtained from palm sugar medium might be related to the nutrient content in the growth medium. Irawan [18], stated that the palm sugar is very rich in glucose which can be used as the energy source of the bacterium. In addition, the palm sugar was also reported to be rich in minerals and other growth stimulants such as calcium, carotene and laktoflavine [8]. Thus, this result suggests that the local material is a potential component to develop a cheap growth medium for *B. amyloliquefaciens* subsp. *plantarum*. However, nutrient contents of the palm sugar medium need to be further studied in order to provide more clues for this present study results.

CONCLUSION

In conclusion, the present study confirms that *B. amyloliquefaciens* subsp. *plantarum* can be grown in media from palm sugar and tofu wastewater. Among two materials, palm sugar medium appeared to give the highest total cell biomass, suggesting that this medium can be used as the alternative replacement of the commercial media such as LB. However, further study needs still to be done regarding the quantity of enzyme production. Furthermore combination of the two local materials should be studied for the growth of the probiotic candidate.

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