Bioconversion of anthocyanin to anthocyanidin from fermented fruit and vegetable waste by *Leuconostoc mesenteroides* as probiotik food material

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Abstract. Organic waste, especially fruit and vegetables is currently a major problem in the world due to serious risks to human health and the environment. On the other hand, fruit and vegetable waste contains anthocyanin (flavonoid of polyphenol) compounds that can produce antioxidant to inhibit oxidation reaction caused by free radicals such as diseases that exist in the digestive tract. it has been an important thing to make a bioproduct solution associated with the animal element’s feed ingredients. The result of this study showed that an anthocyanidin from isolates fruit and vegetable was successfully identified by (i) fermentation time variations used are 0; 1; 3; 5 and 7 days resistant to acid pH 4. The compound isolation process use liquid-liquid extraction method with ethanol 96% by diluting 1:3 in the anaerobic jar (ii) Lactic Acid Bacteria (LAB) were gram positive purple colored and basil shape based on Gram staining (iii) thus, an antimicrobial activity was shown by clear zone with E15 which were 18 mm, 20 mm and 24 mm from 72 hours incubation on nutrient media *Euschericia coli*.

Keywords: Probiotic material, Anthocyanidin, Lactic acid, Bacteria, Organic waste

1. Introduction

Anthocyanin, a derivative of 2-phenylbenzopyran structures, is a common, water-soluble, natural pigment that has a strong antioxidant activity [1]. Anthocyanins have red, purple and blue pigments. The color stability of anthocyanin compositions is determined by the pH or acidity level, and will be more stable in acidic or low pH solutions. Natural dyes that contain poles are included in the class of flavonoids which are divided into polyphenol plants. flavanone, flavanol, flavonoid-3-ol, and flavone are additional flavonoids that are different from the anthocyanin oxidation process [2]. When anthocyanin forms aglycone and releases the glycone it is known as anthocyanidin. Anthocyanidin will be blue in an acidic environment, blue in an alkaline environment and purple in a neutral environment. The color of atocyanidine pigments, one of which depends on the pattern of substitution of hydroxyl groups (-OH) [3].

Indonesia Central Statistics Agency have revealed there are 1976 market units that produce market waste as much as 1,300,000 tons per day in fresh form, and most (60%) is organic waste which has a weakness of high water content of 92.44% [4]. Careless disposal of organic waste can make greenhouse gas emissions where the atmosphere has a different ability to adsrob heat which causes climate change and the environment. Organic waste included vegetable and fruit waste produces many anthocyanin compounds to inhibit oxidation reactions caused by free radicals such as diseases that exist in the digestive tract. Research
on anthocyanin has been carried out on a variety of foods, for example the conversion in anthocyanin to anthocyanidin from black rice bran using *Saccharomyces cerevisiae* [5]. The conducted an anthocyanin research into anthocyanidins by using the extract of fermented red Rosella *Lactobacillus plantarum* [2]. Due to the previous research, the use of anthocyanin from fruit and vegetable was good compound, especially as a probiotic material, in this case using *Lactobacillus Mesenteroides*.

Probiotics are bacteria which when consumed can improve the health of humans or livestock by balancing microflora in the digestive tract. Probiotic bacteria have a positive influence if consumed on the physiology and health of the host. Probiotic bacteria are not pathogenic, genetically stable, and resistant to digestive enzymes [6]. One group of bacteria that acts as a probiotic is *Lactic Acid Bacteria* (LAB) from the bioconversion of anthocyanin to anthocyanidin. LAB is a group of gram-positive bacteria that are able to convert carbohydrates into lactic acid [7]. The use of lactic acid bacteria is because LAB was a food grade microorganism, which is a microbe that is not at risk to health and does not produce harmful toxins in food but has a reverse function that is good for health. LAB can naturally inhibit pathogenic microbes, produce organic acids, reduce the pH of the environment and excrete compounds that can inhibit pathogenic microorganisms such as *H₂*, O₂ diacetyl, CO₂, acetaldehyde, d-isomers of amino acids and bacteriocin [8]. LAB also known as GRAS (generally recognized as safe) status as a microbial producing extracellularly (EPS). EPS from microbes can be divided into two types, namely homopolysaccharides (HoPS) and heteropolysaccharides (HePS). For the synthesis of homopolysaccharides from sucrose, LAB uses a large extracellular enzyme namely glucanukrase or glucosiltransferase (gtf) and fructosysucrase or fructosyltransferase (ift) enzymes. This enzyme is useful for the synthesis of EPS glucans or large molecular weight EPS fructants from sucrose substrates.

*Leuconostoc mesenteroides* are gram-positive bacteria, phylum Firmicutes, which are the lactic acid bacteria that have a coccus or rod shape, do not form spores, not motile, negative catalase and positive oxidation, the optimum temperature is 40 degrees Celsius, as we seen the need for O₂ lactic acid bacteria are anaerobic aerotolerant even in the absence of O₂, these bacteria can grow well in the environments. Lactic acid bacteria have special properties that are able to live at high levels of sugar, salt and alcohol and able to ferment monosaccharides and disaccharides. It has certain characteristics which include: lacking porphyrins and cytochromes, negative catalase, no phosphorylation of electron transport, and only getting energy from phosphorylation of the substrate. Up till now, research on bioconversion of anthocyanins to anthosyanidins from fermented fruit and vegetable waste by *Leuconostoc mesenteroides* is not yet reported. This paper therefore reports the molecular identification of LAB isolated from fruit and vegetable waste and further applied to get their antimicrobial activity. The aim of the present work is to identify an anthocyanin conversion to anthocyanidin for producing food additive probiotic material.

### 2. Experimental

The Materials used in this research are; aquadest, vegetable and fruit waste, 70% ethanol, glacial acetic acid (Merck), concentrated hydrochloric acid, *Leuconostoc mesenteroides*, n-Butanol (Merck), Media Nutrient broth (Merck), media nutrient agar (Himedia, India), MH media (Mueller Hinton), glycerol, spritus, Gram staining kit, slide glass, filter paper, label paper, wrap, aluminum foil, Test bacteria (Euschericia coli), cotton, antibiotics (positive control ) E15.

#### 2.1 Methods

#### 2.1.1 Media Preparation and Making

##### Producing of Nutrient Broth Media (Merck)

A total of 55.15 grams of Nutrient Broth powder were weighed and then put into 2.0 L erlenmeyer, then dissolved in 1.0 L distilled water, heated until homogeneous and sterilized at 121 °C, pressure 15 lb for 15 minutes.

##### Producing of agar nutrient media (Merck)

As much as 28 grams of Nutrient to be weighed in erlenmeyer 2.0 L, then dissolved in 1.0 L until
homogeneous and sterilized at a temperature of 21 °C, put into aquadest, heated to a pressure of 15 lb for 15 minutes, then poured in sterile petridish as much as 15 mL then allowed a few minutes to the media freezes, then stored upside down in the refrigerator.

**Purification of bacterial isolates**
As many as 1 ose of bacterial isolates taken in the glycerol stock was then grown into nutrient agar media by zigzag streaking. Then incubated for 24 hours at 37 °C. Furthermore, the bacteria that grow on the media are purified by growing it back on nutrient agar media and incubated for 24 hours at 37 °C. The bacteria that grow are pure bacteria from isolates.

*Lactobacillus mesenteroides* starter
Microbial growth medium in the form of nutrient broth was weighed as much as 0.16 g broth added with 20 mL aquadest then heated until boiling and transferred to the test tube waiting to cool. After a cold, put 1 ose of Leuconostoc mesenteroides starter bacteria. Then, it is closed tightly and incubated for 24 hours at 37 °C. The starter is ready for further research.

2.1.2 Vegetable and Fruit Waste Sample Extraction
Vegetable and fruit waste samples are crushed first using a blender then weighed as much as 20 g and 500 mL of distilled water is added and heated to boiling. After that, the sample is cooled and taken as much as 100 mL then added with a starter of *Lactobacillus mesenteroides* as much as 20 mL. Then, fermented with a variation of time 1 day, 3 days, 5 days and 7 days in an anaerobic atmosphere. During the fermentation process, pH measurements are carried out per day using a universal pH indicator. The sample is filtered with a Buchner funnel and extracted using a 75% ethanol 96% solvent with a ratio between sample and ethanol which is 1:3. The results of the ethanol extract of vegetable and fruit waste are put into a sample bottle.

2.1.3. Vegetable and Fruit Waste Isolation
**LAB Growth of Vegetable and Fruit Waste**
Start with LAB fermentation results are then isolated with the beginning of the enrichment process, 5 (five) mL samples are mixed into 45 mL Nutrient broth (Merck), it homogenized so as to obtain 1:10 or 10⁻¹ dilution then put into aerobic jar and incubated in an incubator at 37 °C for 24 hours. The planting process is carried out by taking 100 mL of LAB (enrichment process) implanted in jell (Merck) medium, then put in anaerobic jar and incubated in an incubator at 37 °C for 48 hours. A single LAB colony that grows is transferred back into the Nutrient agar (Merck), scratched and put in a desiccator and incubated at 37 °C for 48 hours for purification and morphological identification of LAB will be carried out Gram staining [9].

**Identification of LAB Morphology**
The process of identifying LAB morphology is done in two ways, namely macroscopic and microscopic identification. Macroscopic identification that was observed was the shape, color, edge, and elevation of BAL colonies growing on Nutrient jell medium whereas microscopic identification was cell shape, and physiological identification by Gram staining. The Gram staining procedure begins with the administration of basic dyes, violet crystals, and iodine solution. All bacteria will be colored blue in this phase. The cell is then given alcohol. Gram-positive will still bind violet-iodine crystal compounds, remain blue, Gram-negative color is lost by alcohol. As the final step, counterstains (eg red dye safranin) are added so that colorless Gram negative cells will take on red while positive Gram cells are seen in purple [10].

2.1.4 Biochemical Test
**Acid Resistance Test**
This acid resistance testing was carried out on several variations of the pH of MRS Broth, namely pH 2; 3; 4; 5 and 6. The isolates which had the highest clear zone were grown on the MRS Broth pH 2 to 6. Incubated
overnight at 37 °C, the culture formed was then centrifuged at 6000 rpm for 10 minutes. Antimicrobial test was carried out using jell diffusion method [11].

Antimicrobial Activity Test
The antimicrobial activity test is test bacteria use *Euschericia coli*. The method used was jall well diffusion with E15 control. 3 mL LAB cultures were centrifuged at 6000 rpm for 10 minutes, then the supernatant was used to test antimicrobial activity. 200 mL of test bacteria were put into 20 mL of Nutrient media so that the liquid was still in the temperature range of 40 °C. Poured into sterile petridish. Leave for 30 minutes to harden. E. coli bacteria that have been rejuvenated 24 hours are then taken using sterile cotton and then smeared on the surface of the media. After that the sample paper disc, antibiotics (erythromicin 15), and sterile distilled water discs (negative control) paper were placed on the media using sterilized tweezers. The petridish then wrapped in plastic wrap anaerobically and labeled also incubated in 37 °C for 24 hours. The diameter of the clear zone is measured every day 1, 2 and 3 then compared with the criteria of inhibition strength. Clear zones were formed at 24 hours, 48 hours and 72 hours [11].

3. Result and Discussion
The first step taken in this study was samples preparation of vegetable and fruit waste for bioconversion process of anthocyanin to anthocyanidin by fermented *Leuconostoc mesenteroides*. The vegetable and fruit waste are combined and blended with the addition of distilled water until smooth, the purpose of refinement of the sample is to minimize surface pores so that the surface area is larger which causes the solvent to more easily penetrate into the cell and withdrawal of chemical compounds contained in the sample more leverage. The finely blended sample is then heated where the purpose of the heating is to attract the desired compound, anthocyanin, which based on its physical properties anthocyanin that dissolved in warm water. The sample was cooled and then the volume was taken 100 mL, fermented with the addition of 5 mL *Leuconostoc mesenteroides* starter in an anaerobic beaker covered with plastic wrap.

The fermentation process is carried out aiming to increase the biological activity of the compounds contained in extracts of vegetable and fruit waste. Fermentation is a process of chemical change in an organic substrate through the activity of enzymes produced by microorganisms. The reason of using *Leuconostoc mesenteroides*, which is heterofermentative bacteria and these bacteria have the ability to live in high levels of sugar, salt or alcohol [12]. The type of fermentation carried out is lactic acid fermentation. During the fermentation process pH measurement was carried out on the extracts of vegetable and fruit waste which aims to determine the environmental conditions of the pH to pay attention to the stability of anthocyanin. The results of pH measurements from vegetable and fruit waste can be seen in Table 1.1

| No | Variation Time | pH  |
|----|----------------|-----|
| 1  | 0 day          | 5.0 |
| 2  | 1 day          | 4.0 |
| 3  | 3 day          | 4.0 |
| 4  | 5 day          | 4.0 |
| 5  | 7 day          | 4.0 |

Vegetable and fruit waste extracts with a variation of fermentation time have an initial pH value of 5.0 and at a fermentation time variation of 1, 3 and 5 days the pH has decreased which is constant with a value of 4.0 accompanied by the color of the solution that turns to turbid. Several factors that affect the color change in the sample, namely fermentation time, storage time, temperature, water content in the sample, and exposure to light, where the longer the fermentation, the starter given to the sample will be higher in its activity in remaking glucose into lactic acid so causes an increase in acidity and the pH becomes decreased or more acidic [13].
3.1 Vegetable and Fruit Waste Isolation

An isolation of fruit and vegetable waste was done by fermentation for 72 hours. The organic acids of the fermentation product are the hydrolysis of fatty acids and also as the result of bacterial activity. The amount of energy produced is 2 ATP, with its chemical reaction $\text{C}_6\text{H}_{12}\text{O}_6 \rightarrow 2\text{C}_2\text{H}_5\text{OH} + 2 \text{CO}_2 + 2 \text{ATP} \Delta H = -118 \text{kJ per mole}$. The result of fermentation has been dilution $10^{-7}$. This is done to enrich the number of bacteria that will grow in the media. The total number of these colonies met with the criteria for food as probiotics according to Food and Agriculture Organization (FAO) and World Health Organization (WHO) which stated that the number of colonies of probiotic bacteria in a food must have a colony count of $10^6 - 10^8 \text{CFU/mL}$.

The colony was then tested macroscopy to determine the LAB that formed. As a result, gram staining shows LAB in the form of bacilli with a variation of gram-positive bacteria which is a microbial that has a layer of peptidoglycans (molecules consist of amino acids and sugars). Following the Gram test, when violet-iodine crystal compounds are added, the LAB on the glass remains blue, while when given counterstains (eg red dye safranin) it is very clear that it changes purple. Microscopically, the shape of the LAB in this colony is shown in (Figure 1).

![Figure 1. Shape of the LAB colony on Gram Staining](image)

3.2 Biochemical Test

Acid resistance test was carried out on several variations of the pH there are 2, 3, 4, 5 and 6. The isolates which had the highest clear zone were grown on the MRS Broth put inside. Incubated overnight at 37 °C, after some hydrochloric acid added to the culture then centrifuged at 6000 rpm for 10 minutes. It is seen that media with pH 4 very resistant to acidity are supported (Figure 2). The more turbid broth shows the greater the potential for LAB to live at pH in its culture.

![Figure 2. pH variation 2; 3; 4; 5 and 6](image)

An antimicrobial activity test in this case was used *Euschericia coli* and E15 (erythromycin) control in microflora enhancement was well administered. It not only actively inhibits the development of Euschericia coli which is even dangerous if there is a large amount on digestive track but also increases the productivity of healthy livestock. Results obtained from antimicrobial activity test (Figure 3)
The results of antimicrobial activity test obtained in (Figure 3) for sample fruit and vegetable waste at 24 hours, 48 hours and 72 hours shows a clear zone. Samples were obtained at each treatment time of 18 mm, 20 mm, and 24 mm. Results of the clear zone formed because the presence of bacteriocins that inhibit the growth of pathogenic bacteria that formed around the colony, which is on 11 mm, 13 mm, and 14 mm. From the data, we conclude that the LAB of the isolate sample is one of the requirements in the development of probiotics food material for livestock ingredients. Bioconversion of anthocyanin to anthocyanidin has occurred during the process of extraction and fermentation [14] because anthocyanidin plays an active role in the formation of good bacteria in the intestine by presence of LAB [15]. As simple as reducing existing organic waste and supporting a healthy environment, this additive is very good related to the effectiveness of the experiment.

**Enzyme activity test**

In measuring the activity of protease enzymes from a sample using a UV-VIS spectrophotometer method with several phases of action before the enzyme test first produced an enzyme by taking 1 one a protease bacterium insulation which is obtained, then inoculated into a liquid medium that is nutrient broth six ml. The supernatant obtained is a crude enzyme extract to be used for further determining crude extracts from bacterial culture will be analyzed which have been centrifuged at 5000 rpm for 10 minutes. The blue color in the sample is caused by the use of Folin-Ciocalteau reagents. Enzyme activity in vegetable and fruit waste samples 1, 2, and 3 days respectively were 9 mm, 12 mm, and 15 mm which enzyme specific activity was calculated as follow result 1,0286 unit/mg from value 148,6408 ppm as X at the spectrometry data.

**Phytochemical screening analysis**

Phytochemical test results from extracts of vegetable and fruit waste that are positive containing flavonoids and phenolics are marked by changes in the color of the extract samples. In the flavonoid test the color changes from the sample to yellow. Flavonoid compounds have the ability to inhibit bacterial growth by damaging the permeability of the bacterial wall. The phenolic test in the sample was carried out using FeCl3 which was put into the extract sample from the evaporation. Positives contain phenols if they produce green, red, purple, blue or deep black colors. The samples appear to produce a deep black color so that they qualitatively contain phenols. Also it shown in spectra, the incisive peak on 3273.06 cm\(^{-1}\) which is thought to be absorption from OH groups. Then Whereas the carbonyl group (\(-\text{C} = \text{O} +\)) which is a common characteristic of anthocyanin compounds is indicated by the absorption in the region of wave number 1643.85 cm\(^{-1}\). C-O carbonyl appears in absorption of 1014 cm\(^{-1}\). All of this data conclude that the LAB of the isolate sample is one of the requirements in the development of probiotics food material for livestock ingredients. According to Bagchi 2004, bioconversion of anthocyanin to anthocyanidin has occurred during the process of extraction and fermentation, this clear zone is very supportive statement because anthocyanidin plays an active role in the formation of good bacteria in the intestine by presence of LAB [15]. As simple as reducing existing organic waste and supporting a healthy environment, this additive is very good related to the effectiveness of the experiment.
Figure 4. Phytochemical result of two compound, a; flavonoid, b; phenolic

4. Conclusion
In conclusion, the results of LAB identification of fruit and vegetable waste was basil shaped with the total number of these colonies that included the criteria as food material for probiotics, supported by the temperature resistance identified at pH 4. Erythromicin (E15) as the control showed an antimicrobial activity in larger 14 mm on 72 hours prove anthocyanin has been converted to anthocyanidin.

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