PENGAYAAN ASAM FOLAT PADA JUS PISANG FERMENTASI MENGUNAKAN KULTUR BAKTERI ASAM LAKTAT

(Folic Acid Enrichment of Fermented Banana Juice with Lactic Acid Bacteria)

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ABSTRAK. Asam folat (Vitamin B9) terlibat dalam berbagai metabolisme. Kekurangan folat bertanggung jawab atas beberapa masalah kesehatan. Fermentasi bahan pangan untuk menghasilkan minuman kaya asam folat menggunakan bakteri asam laktat (BAL) merupakan upaya untuk mengatasi masalah kesehatan akibat defisiensi folat. Penelitian ini bertujuan untuk mengetahui pengaruh fermentasi jus pisang menggunakan BAL terhadap kandungan asam folat jus pisang. Jus pisang difermentasi selama 48 jam pada suhu 37°C dengan kultur tunggal *Lactobacillus plantarum* dan kultur campuran BAL yaitu *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Bifidobacterium bifidum* dan *Bifidobacterium breve*. Perubahan karakteristik asam folat diamati setiap 8 jam selama 0-48 jam fermentasi. Selain itu, diamati pula perubahan pH, total asam, protein terlarut dan gula pereduksi. Identifikasi monomer asam folat dilakukan pada kondisi fermentasi paling optimum dengan menggunakan LC-MS. Hasil penelitian menunjukkan bahwa fermentasi dapat mempengaruhi kandungan asam folat, pH, total asam, gula pereduksi dan protein terlarut dari jus pisang. Selama fermentasi, pH sampel menurun diikuti dengan peningkatan total asam. Nilai gula pereduksi dan protein terlarut cenderung berfluktuasi. Kondisi optimum peningkatan kandungan asam folat diperoleh pada fermentasi menggunakan mix kultur LAB selama 32 jam dengan kandungan asam folat 34,07 mg/mL, pH 4,00, total asam 0,51%, gula pereduksi 119,17 mg/L dan protein terlarut 0,43 mg/mL. Identifikasi monomer asam folat pada kondisi optimum fermentasi didominasi senyawa dengan berat molekul 442,49 Dalton (Da).

Kata kunci: asam folat, bakteri asam laktat, fermentasi, pisang

ABSTRACT. Folic acid (Vitamin B9) involved in numerous metabolisms. Folate deficiency is responsible for some negative health problems. The fermentation of foodstuffs to produce rich content of folic acid beverages using lactic acid bacteria (LAB) is an effort to overcome the health issues. This study aimed to determine the effect of banana juice fermentation using LAB on the folic acid content of the banana juice. Banana juice was fermented for 48 hours at 37°C with monoculture of *Lactobacillus plantarum* and mixed LAB cultures of *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Bifidobacterium bifidum* and *Bifidobacterium breve*. The changes of folic acid characteristics were observed every 8 hours during 0 – 48 hours of fermentation, as well as the change in pH, total acid, soluble protein, and reducing sugar. The identification of folic acid monomers was conducted at the most optimal condition of the fermentation by using LC-MS. The result showed fermentation affect the folic acid content, pH, total acid, reducing sugar and soluble protein of banana juice. During fermentation, pH of the sample decreased with the increasing of total acid. Reducing sugar and soluble protein content tended to fluctuate. Optimal condition for folic acid enhancement was obtained by 32 hours of fermentation with mixed LAB, in which it yielded folic acid 34.07 µg/mL, pH 4.00, total acid 0.51%, reducing sugar 119.17 mg/L, dissolved protein 0.43 mg/mL. Folic acid identification on the optimal condition revealed that it was dominated by compound with a molecular weight of 441.49 Dalton.

Keywords: banana, fermentation, folic acid, lactic acid bacteria

1. INTRODUCTION

Folate or B9 vitamin (one of the B vitamins, referring to all derivatives of folic acid) is a water-soluble micronutrient involved in numerous biological metabolisms in human. It participates in the reaction of carbon transfer in particular pathways of metabolism, namely purine and pyrimidine biosynthesis and the interconversion of amino acid (Forssnet *et al.*, 2000; Saubade *et al.*, 2017). Folate is an essential nutrient that cannot be synthesized by the human body, thus exogenous supply (food diet or supplementation) of folate is necessary to prevent folate deficiency. Folate deficiency is responsible for such negative health problems as anemia, neural tube defects (NTDs), Alzheimer's disease (AD), coronary heart disease, osteoporosis, increasing risk of breast and colorectal cancer, poor cognitive function and impaired hearing. The recommended daily intake (RDI) of folate adult is 200 – 400 µg whereas for pregnant woman is 400 – 600 µg. Folate presents in nuts (peanuts, hazelnuts, beans), vegetables (spinach, broccoli, cauliflower), fruits (banana, orange, apple) and fermented milk (LeBlanc *et al.*, 2007; Saubade *et al.*, 2017). Nevertheless, cases of folate deficiency are still prevalent in adults. In addition, food fortification with synthetic folate (folic acid) induces another issue, which is a risk of obscuring anemia caused by vitamin B12 deficiency and delay proper treatment (Morris & Tangney, 2007).

Fermentation, oftentimes reported help improving the nutritional value and vitamins of the fermented foodstuff, including folate. The favored nutrition in the foodstuffs improves due to microbial activity during fermentation. Fermentation is a safe, natural, and efficient method of folate fortification (Laio *et al.*, 2012; Wegkamp., 2008). Lactic acid bacteria (LAB) is identified as a gram-positive, non-sporeforming, microaerophilic, aerotolerant, which produces lactic acid as the end product of carbohydrate fermentation (Papadimitriou *et al.*, 2016). It is widely used in the production of fermented foodstuffs owing to their capability to produce unique taste, smell and texture (Rattanachaikunsopon & Phumkhachorn, 2010). LAB is also known to be a generating antimicrobial substances, such as bacteriocin, organic acid, hydrogen peroxide, diacetyl and carbon monoxide (Vieco-Saiz *et al.*, 2019). Several kinds of LAB from genus *Lactobacillus* and *Bifidobacterium* are reported to be capable of synthesizing folate. *Lactobacillus plantarum* is

known for generating folate, although in a low concentration, under the circumstance where it has grown in folate-free medium with a particular chemical composition (Sybesma *et al.*, 2003). *Lactobacillus acidophilus* enhances folate concentration in fermented milk (Lin & Young, 2000). *Lactobacillus lactis*, *Bifidobacterium bifidum* and *Bifidobacterium breve* as well are able to synthesize folate in milk (Crittenden *et al.*, 2003; Pompei *et al.*, 2007). Although, several kinds of LAB instead consume available folate in the media for their growth. The growth medium it self regulates the ability of LAB to synthesize folate. The proper LAB strain and growth medium consequently contribute to the fortification of folate in fermented foodstuffs (Saubade *et al.*, 2017).

Fruit juice is one feasible substrate for LAB growth owing to its nutritional value. Juice provides the benefits of natural nutritional value and the added value of probiotic. Banana is one of the most eminent fruits in the world. It can be processed into various products, including fruit juice (Singh *et al.*, 2016). Banana is rich in starches, sugars, vitamin A, vitamin C, folate, potassium, calcium, sodium, and magnesium (Ashokkumar *et al.*, 2018). Thus, the examination of banana juice fermentation with lactic acid bacteria is convenient approach as was we seek for a food diversification effort to prevent such prevalent health issues related to folate deficiency. This research aimed to evaluate the effects of banana juice fermentation with two types of starters: mixed LAB culture (*L. acidophilus*, *L. casei*, *L. plantarum*, *B. bifidum*, and *B. breve*) and *L. plantarum* monoculture on folic acid content of banana juice (*Musa acuminata* Linn).

2. METHOD

The materials used are fresh bananas of *Musa acuminata* Linn (locally known as Pisang Muli), sucrose and skim milk were bought from local market, mixed cultures of LAB (consisting 5 probiotics: *Lactobacillus acidophilus*, *Lactobacillus plantarum* HAC01, *Lactobacillus casei*, *Bifidobacterium bifidum* and *Bifidobacterium breve*) obtained from Atogen Korea and single culture of *Lactobacillus plantarum* obtained from Gadjah Mada University Yogyakarta. The other materials used were 3-aminophenol, hydrochloric acid (4M), sodium nitrite, sulfamic acid, glucose (Sigma Aldrich, USA), MRSB, distilled water and necessary chemicals. Chemicals used were analytical grade.

The equipment included measuring scale (Mettler Toledo, Switzerland), analytical balance (Kern ABJ 320-420, Balingen), blender (National BL-T9A, Indonesia), homogenizer (Ultraturax T50, China), autoclave (HL36Ae, Japan), centrifuge (Ependorf AG, USA), vortex (thermolyne maximix plus TM., USA), spectrophotometer (Agilent Technologies Cary 60 UV-Vis, USA), pH meter (Eutech Instruments pH 700, Singapore), hotplate, laminar air flow, autoclave and other glass equipment.

Fermented Banana Juice Production

Fresh bananas were peeled, cut, added with distilled water (banana:distilled water = 1:3) and mixed by using blender for one minute. Banana juice was fortified with 5% skim milk (w/v) and 10% sucrose (w/v) for LAB growth. Banana juice was further homogenized for 15 minutes at 8000 rpm by using homogenizer (Ultraturax T50, China). Fermentation took place in 25 x 150 mm test tubes. Each tube containing 40 mL banana juice, was sterilized in autoclave for 15 minutes at 121°C. Sterilized banana juice was inoculated with 5% of *L. plantarum* monoculture (v/v) or 5% mixed LAB culture (v/v). The inoculum was prepared by growing 5% starter broth of *L. plantarum* (v/v) or 5% starter broth of mixed LAB culture (v/v) in MRS broth and was incubated for 48 hours at 37°C. Fermentation of banana juice was carried out in an incubator at 37°C in a time of 0–48 hours, with an observation interval of 8 hours. Banana juice without culture addition was used as a control.

Folic Acid Content

Folic acid analysis of banana juice was conducted by using spectrophotometry method (Kumlueharn *et al.*, 2012). Test solution, containing 1 mL of folic acid standard or samples, 1 mL of 4M HCl, 1 mL of 1% sodium nitrate (w/v), 1 mL of 1% sodium nitrite (w/v), 1 mL of 1% sulfamic acid (w/v) and 1 mL of 1% 3-aminophenol (w/v), was prepared. Test solution was incubated for 2 hours at 37°C in a dark condition. The absorbances was measured by using UV-Vis spectrophotometer at 460 nm.

Determination of pH, total acid, reducing sugar and dissolved protein

Digital pH meter (Eutech Instruments pH 700, Singapore) was used for measuring the pH of banana juice. Titratable acidity of banana juice was measured by titration using 0.1M NaOH and the value was expressed in lactic acid percentage (Yang *et al.*, 2012). Reducing sugar value was determined by using Smogyi – Nelson method (Smogyi, 1952). Glucose was used as the standard for measuring reducing sugar. Soluble protein content was identified by using Lowry's method, with Bovin Serum Albumin (BSA) was used as the standard (Lowry *et al.*, 1951). All tests, except pH test, were conducted with two repetitions.

Folic acid identification by using LC-MS

The identification of folic acid monomer using LC-MS (positive ion mode) was performed on the fermented banana juice with the highest improvement of folic acid (optimal condition). As much as 100 µL fermented banana juice was diluted with 900 µL methanol. The mixture was then centrifuged and the filtrate was used as the sample. The analysis was carried out by employing Mariner Biospectrometry integrated with Q-tof mass spectrometer (MS) through ESI (electrospray ionization) system with scan mode 430 - 450 sqm at 140°C. The column of C18-RP18 Supelco (250 x 2 mm, 5 µM particle size) was employed in the analysis of folic acid using LC (Hitachi L 6200). Solvent used in the analysis was methanol containing 0.3% acetic acid. The flow rate was set at 1 mL/min. The injected sample had a volume of 5 µL (Eichhorn & Knepper, 2001).

3. RESULT AND DISCUSSION

Characterization of Unfermented Banana Juice

The muli bananas are widely consumed as a good source of nutrition. This fruits are very prone to deterioration during ripening process. The muli banana are usually consumed fresh, but can be processed and consumed in form of juice. Banana juice is viscous, turbid, pretty sour and yellow in color. The pH, total acid, reducing sugar, dissolved protein and folic acid content of unfermented banana juice (banana juice without LAB culture addition) were displayed in Table 1.

Table 1. Characterization of unfermented banana juice

Sample	pH	Total acid (%)	Reducing sugar (mg/mL)	Dissolved protein (mg/mL)	Folic acid (µg/ml)
Non fermented banana juice	4.53	0.3	40.04	0.50	15.67

pH and Total Acid of Fermented Banana Juice

Fermentation with lactic acid bacteria reduced pH and increased total acid. The pH value of unfermented banana juice (4.53) is higher than fermented banana juice (Figure 1a and Table 1). Fermented banana juice with mixed LAB culture had pH value ranging from 4.21 to 3.97 for 0 to 48 hours fermentation. Meanwhile, fermented banana juice with *L. plantarum* had slightly lower pH, ranging from 4.17 to 3.92 for 0 to 48 hours of fermentation. Total acid in unfermented juice was 0.3%, lower than both fermented samples (Figure 1b and Table 1). For 0 to 48 hours of fermentation, fermentation with mixed LAB culture yielded 0.33 to 0.59% total acid, whereas fermentation with *L. plantarum* yielded 0.42 to 0.62% total acid. The

decrease of pH and increase of total acid during fermentation was a result of lactic acid production by LAB. LAB consume carbohydrate as its energy source and utilize endogenous carbon source as electron acceptor. Based on the product of fermentation, LAB is categorized into homofermentative and heterofermentative bacteria. Homofermentative bacteria produce solely lactic acid as their product of fermentation, whereas heterofermentative bacteria ferment glucose with lactic acid, acetic acid, alcohol, and carbon dioxide as their products (Mokoena, 2017). *L. plantarum*, *L. casei*, *B. bifidum*, and *B. breve* are heterofermentative bacteria while *L. acidophilus* are homofermenters (Carr *et al.*, 2002; Pokusaeva *et al.*, 2011).

(a)

(b)

Figure 1. a) pH of fermented banana juice, b) total acid of fermented banana juices

Reducing Sugar and Dissolved Protein of Fermented Banana Juice

Reducing sugar is a highly water-soluble sugar, acting as reducing agent for its reactive double bond in free ketone group (such as fructose) or free aldehyde group (such as glucose and maltose) (Halford, 2019). The amount of reducing sugar of fermented banana juice during the experiment tended to fluctuate (Figure 2a). At hour-48, banana juice fermented with *L. plantarum* had lower reducing sugar than the unfermented banana juice (40.04 mg/mL). Lactic acid bacteria consume sugars in the media to generate lactic acid (Li *et al.*, 2019). In contrast, the banana juice fermented with mixed LAB culture yielded higher reducing sugar than unfermented banana juice at hour-48 (Figure 3 and Table 1). This outcome was

presumably due to the high concentration of lactic acid bacteria, resulting in inefficiency of the bacteria in metabolizing sugars, also as well due to the physical and chemical properties of the sample. As fermentation occurred for 48 hours, dissolved protein tended to fluctuate (Figure 4). The change might happen due to the production of secondary metabolite like antimicrobial peptide (bacteriocin) during fermentation (Mokoena, 2017). LAB also generates proteolytic enzyme to breakdown peptide bond of protein chain (Maryati & Susilowati, 2018). They consume fermentable amino acid, peptide, and protein during fermentation for its growth; it is presumed to be the answer to this fluctuating dissolved protein result.

Figure 2. a) reducing sugar of fermented banana juices, b) dissolved protein of fermented banana juices

Folic Acid Content of Fermented Banana Juice

The optimal condition in terms of obtaining the highest folic acid was observed at hour-32 for both treatments. Figure 3 displays that folic acid content at hour-32 in fermented banana juice with mixed LAB was increasing higher than the *L. plantarum* monoculture. The bacteria used in the experiment (*L. acidophilus*, *L. lactis*, *L. plantarum*, *B. breve*, and *B. bifidum*) are bacteria that can produce natural folate depending on the fermentation condition and medium types;

therefore, the use of mixed culture of LAB yielded higher folic acid than a monoculture (Saubade *et al.*, 2017). The folic acid content of fermented banana juice with mixed and monoculture at hour-32 is also higher than unfermented banana juice (15.67 µg/ml). It shows that fermentation of banana juice using lactic acid bacteria can increase the folic acid content in banana juice. Lactic acid bacteria used in this study able to utilize banana juice as medium to produce natural folate.

Figure 3. Folic acid content of fermented banana juices

Folic Acid Identification using LC-MS

The identification of monomer in banana juice fermented in optimum condition (32-hour) by using LC-MS aimed to prove the presence of folic acid in the juice. Folic acid presents naturally and synthetically. Folic acid's chemical structure is constituted of three moieties: a p-aminobenzoic acid (PABA) residue, glutamic acid (Glu) residue and pterin residue. The pterin ring conjugates to PABA by a methylene bridge and via peptide bond, joins to a glutamic residue, forming folic acid (Saubade *et al.*, 2016). Figure 4a and 4b display the chromatogram and mass spectrum of folic acid standard analyzed by LC-MS in positive ion mode operation. As shown in Figure 4a, chromatogram of folic acid standard shows a peak (T1.9) between minute 0 – 10 (relative intensity of 100%). Mass spectra reading shows T1.9 peak is dominated by a compound with molecular weight [M] of 441.42 (relative intensity of 100%) between

m/z of 439.6 – 445.9. According to Bansal *et al.* (2018), folic acid has a molecular weight [M] of 441,4 Dalton, suggesting that the compound with molecular weight [M] = 441.42 was folic acid. The compound in LC-MS assay was identified based on its MW with fragmentation possibilities of M^+ , M^+Na^+ , $2M^{++}$ or $2M^+$, Na^+ . Fragmentation pattern are formed due to ionization and as a consequence of LC-MS sensitivity to the eluent. Chromatogram of fermented banana juice (Figure 4c) indicated a peak T2 between retention time of 0 and 10 minute (relative intensity of 100%). Figure 4d exhibits spectrum T2 (at m/z = 442 – 443) consisting of 9 folic acid monomers and dominant monomer with MW [M] of 441.49 Da. These results proved the presence of folic acid in the fermented banana juice with mixed culture of LAB for 32 hours. Therefore, this product is a reliable natural source of folic acid as functional food.

(a)

(b)

Figure 4. (a) Chromatogram LC-MS of standard folic acid, (b) mass spectra of standard folic acid

Figure 4. (c) chromatogram of fermented banana juice using LAB mixed cultures at optimum condition, (d) mass spectra of fermented banana juice using LAB mixed cultures at optimum condition.

4. CONCLUSION

Lactic acid fermentation using mixed LAB culture (*L. plantarum* HAC01, *L. acidophilus*, *L. casei*, *B. bifidum*, and *B. breve*) and a monoculture of *L. plantarum* is an affordable and practical technology to enhance natural folic acid content of banana juice. Optimal condition for folic acid enhancement was obtained by 32 hours of fermentation with mixed LAB culture, in which it yielded: folic acid 34.07 µg/mL, pH 4.00, total acid 0.57%, reducing sugar 119.17 mg/mL and dissolved protein 0.43 mg/mL. Folic acid identification on the optimal condition revealed

that it was dominated by compound with a molecular weight [M] of 441.49 Dalton.

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