Fermentation time difference of nixtamalized horse dent corn (Zea mays var. indenata) by Bifidobacterium bifidum and Bifidobacterium brevis as source of natural folic acid

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Abstract. Bifidobacterium sp. as microbes has potential role in fermentation of nixtamalized horse dent corn (Zea mays var. indentata) to degrade complex components into folic acid-rich corn biomass. Fermentation process on both nixtamalized yellow corn and white corn by Bifidobacterium brevis and Bifidobacterium bifidum as substrat of A, B, C and D were conducted at concentration of corn folic acid inoculum 40% (w/w) and 37 °C for 0, 8, 16, and 24 hours, respectively. Based on dissolved protein yielded, the experiment result showed that the best result of optimization in fermentation of both nixtamalized yellow corn (biomass B) and white corn (biomass D) was achieved by using inoculum of B. bifidum for 16 hours with composition of folic acid of 213.58 and 297.72 µg/mL, total solids of 21.14 and 21.07 %, dissolved protein of 0.42 and 0.39 mg/mL, reducing sugars of 34.2 and 37.8 mg/mL, total sugars of 104.7 and 98.6 mg/mL, total acids of 0.37 and 0.44%, N-amino of 0.28 and 0.26 mg/g, and pH 4.82 and 4.49, respectively. In this condition, biomass of B. and biomass of D indicated domination of folic acid monomer with molecular weight (MW) 442.29 and 442.59 Dalton (Da.) at relative intensity 100%, particles size of 1115.1 nm and 1075.7 nm, and particle index of 0.827 and 0.849, respectively. Meanwhile, volatile compounds were dominated by 2,3-butanediol of 4.46 and 10.65%, palmitic acid of 7.63 and 8.26%, octadecenoic acid of 6.31 and 9.5%, lactic acid of 2.37% and 0.53%, respectively.

1. Introduction
Corn (Zea mays L.) is one of the best staple cereal foods as source of polysaccharides for energy and build of body. Due to high protein content (7 – 9%), corn had proven as potential source of folic acid and other bioactive components (beta caroten, etc.). Folic acid is an essential nutrient which is required for period of replication and growth of the cells on the various body functions in order to maintain life, in which it has important role as co-enzyme in synthesis of methionines, purines and pyrimidines. Co-enzyme of folate has important role as donors and acceptors in the one-carbon units in various important transfer reactions needed in synthesis of many important metabolism of nucleic acids and amino acids [1]. As sources of folates, corn is a potential substrate for growing Lactic Acid Bacteria (LAB), particularly B. bifidum and B. brevis through fermentation process. In generally, mechanism in forming folic acid through fermentation of LAB is possibility caused by its occurrence of biosynthesis de novo. It had been known that folic acid is a group of water soluble B-vitamins (vitamin B₉), consisting of a pteridine ring originating from dihydropterin pyrophosphate (DHPPP) and a para-aminobenzoate (p-ABA) and a g-linked tail with one or more L-glutamates produced by
plant [2] and as a precursor in forming folic acid. Biosynthesis will subsequent form 7,8-dihydropterate (DHP) and dihydrofolate and becomes co-factor of tetrahydrofolate (THF) and THF-polyglutamates [3].

Figure 1. Structure of folic acid

Nixtamalized corn is get by treating modification using Calcium Hydroxide (Ca(OH)2) to bind and adsorb folic acid. Process of Ca(OH)2 causing starch gel is able to trap and maintain folic acid [4]. Using B. bifidum and B. brevis are not only functioning as agent being able to form vitamin B9 (folic acid), but also it is caused by ability in degrading sucrose to organic acids, particularly lactic acid followed by less amount of CO2, taste and specific aroma like yoghurt. Fermentation time is a important factor, in which optimization on process is caused by inoculum concentration, type of Bifidobacterium sp., type of corn commodity, and riched sucrose and and riched skimmed milk. This matter is also effected by intrinsic, such as enzymatic activity of β-D-Galactosidase [5]. This fermentation process enables to affect on composition of biomass, folic acid monomer, dominant volatile compounds, both particle size and distribution of particle size of biomass, and evaluation index of hedonic on organoleptic.

This experiment work was carried out to find out the best fermentation time at fixed condition (37 °C, 40% inoculum concentration) from nixtamalized horse dent yellow and white corn using B. bifidum and B. brevis on composition, characteristic of folic acid monomer, volatile compounds, particle size and particle size distribution as source of natural folic acid.

2. Materials and Methods

Main materials used in this experiment activity were distilled water, two dent corn types (dry yellow and dry white (Zea mays var. indenata) procured from a domestic market, culture of B. bifidum and B. brevis (FNCC-UGM), DeMann Rogosa Sharpe B (MRS B) medium (E.Merck), Ca(OH)2 (E.Merck), standard folic acid (E.Merck), skimmed milk (Benato, USA), sucrose (local), standard folic acid (Aldrich), hydrochloric acid (E.Merck), sodium nitrite (E.Merck), and methanol. All the chemicals used were of reagent grade procured locally and used without further purification. distilled water, chemicals used in this process and analysis were standard folic acid (Aldrich), hydrochloric acid (E.Merck) and sodium nitrite (E.Merck).

Main equipments used in this experiment activity were Balance (Fujitsu, Japan), series of nixtamalization process system in laboratory scale : pan (local), blender (local, National), a series of microbiology process equipments, system of laminar flow chamber (local), incubator (local). Main instruments used to analyse is were UV-vis Spectrophotometer (Model RF-550, Shimadzu, Japan), Liquid Chromatography coupled with Mass Spectrometer (LC-MS) (Mariner Biospectrometry) with LC (Hitachi L 6200), Particle Size Analysis using PSA (Particle Size Analyzer) Coulter SZ 100 (Horiba Nano Partica-Backman), and Gas Chromatography-Mass Spectrometry (GC-MS) (Shimadzu, Japan).

This research was conducted at the Research Center for Chemistry, Indonesian Institute of Sciences, South Tangerang. This research was conducted in March-November 2020. The experiment activities were conducted through fermentation of substrates of nixtamalized yellow corn by corn folate inoculum of B. brevis (A), nixtamalized yellow corn by corn folate inoculum of B. bifidum (B), nixtamalized white corn by corn folate inoculum of B. brevis (C), and nixtamalized white corn by corn folate inoculum of B. bifidum (D) at concentration of 40% (v/w, dissolved protein) and 37 °C for 0, 6,
12 and 24 hours described as biomasses of A, B, C, and D, respectively. Analyses were performed on dissolved protein (Lowry method) [6], total solids (Gravimetric method), reducing sugars (Somogyi-Nelson method), total sugars (Phenol – Sulphate method) [7], N-Amino [8], and pH. Identification of folic acid monomer and folic acid concentration (LC-MS) [9], particle size distribution (PSD) [10], and volatile compound (GC-MS) [11]. Process and analysis were conducted in duplicate. Data processed in this description were based on the result of average analysis.

2.1. Nixtamalization process of corn
A number of both yellow corn and white corn from type of horse dent (*Zea mays indenata*) was cleaned, separated from impurities, washed, and steep in water on a 1 : 4 ratio for 18 hours followed by adding both Ca(OH)₂ at 20% for yellow corn and 30% for white corn [4]. Further, cooking was carried for 60 minutes (yellow corn) and 30 minutes (white corn), cooling at room temperature, and pulverizing by blender at maximum speed to get pulp of corn nixtamalized.

2.2. Preparation of stock culture and corn folate inoculums of *B. bifidum* and *B. brevis*
Preparation of stock culture was carried out by weighing 15.6 g of MRS B medium, adding 300 mL of aquadest, autoclaving at 121 °C for 15 minutes, cooling, inoculating each starter of *B. bifidum* and *B. brevis* and 1% of (w/v MRS B), incubating at 37 °C for 24 – 36 hours. Preparation of corn folate inoculum was conducted by mixing a number of pulp of corn nixtamalized with 1 part of yellow corn and 1 part of white corn, and 4 parts of water in order to get substrates. On each substrate was carried out by adding sucrose (10%, w/w) and skim milk (10%, w/w), autoclaving at 121 °C for 15 minutes, and cooling at room temperature. Further, pulp of yellow corn nixtamalized was inoculated by cultures of *B. brevis* and *B. bifidum* 40% (v/w, dissolved protein of nixtamalized corn) and fermented at 37 °C for 8 hours in order to get inoculum of corn folate A and inoculum of corn folate B. Similar treatment was done on pulp of white corn nixtamalized by using culture pulp of white corn nixtamalized by using and cultures of *B. brevis* and *B. bifidum* in order to get inoculum of corn folate C and inoculum of corn folate of D.

2.3. Fermentation process of nixtamalized corn
Fermentation was performed by preparing a number of pulp yellow corn nixtamalized and pulp of white corn nixtamalized (400 mL), adding powder milk (10% w/w), sucrose (10% w/w), autoclaving at 121°C for 15 minutes, cooling at room temperature, inoculating by inoculum of corn folate A and inoculum of corn folate B on pulp of yellow corn nixtamalized and inoculum of corn folate C and inoculum of corn folate D with concentration of 40% (w/w, dissolved protein of pulp corn nixtamalized ), fermentation at 37 °C for 0, 8, 16, and 24 hours to get biomasses of A, B, C, and D, respectively.

2.4. Identification and analysis of folic acid by means of LC-MS
Suitable aliquots of samples being contained in biomass and standard folic acid are used in analysis. Oligomer was analyzed through a LC-MS using Mariner Biospectrometry. LC system was integrated with Q-TOP MS through Electrospray Ionization (ESI) system, in which scan mode was performed in a range of m/z 100 – 1200 at 140 °C. LC (Hitachi L 6200) was carried out on a 15 mm × 2 mm C18 (RP 18) Supelco column and particles size of 5 μm. Types of solvent were a mixture of 80 parts of methanol and 20 parts of water with flow rate 0.1 mL/minute and injection volume of 5 uL [9]. To know concentration of sample folic acid, it is made a curve of standard folic acid in various concentrations followed by identifying samples by LC-MS. Recovery of abundance data at each peak is entered to standard curve from concentration (ug/mL) vs abundance/area in order to get an equation of linear regression from standard. From samples abundance is conducted by plotting in standard folic acid in order to know its concentration.
3. Results and Discussion

3.1. Characteristics of Materials

Compositions of corn, nixtamalized corn, and pulp of corn nixtamalized showed differences in compositions of material, as shown in Table 1. White corn, nixtamalized corn, and pulp of white corn nixtamalized display higher dissolved protein, namely 0.88, 0.22, and 0.20 mg/mL compared to similar components for yellow corn. Dissolved protein is an indicator about the presence of folic acid because of glutamic acid concentration in one of the structures. On total solids, total sugars, and reducing sugars are caused by interaction result on nixtamalization treatment involving time, temperature, and concentration of Ca(OH)2 on difference in variety and post harvesting treatment [4].

Table 1. Composition of materials in fermentation of nixtamalized corn as source of natural folic acid

| Type of material                  | Dissolved protein (mg/mL) | Total solids (%) | Total sugars (mg/mL) | Reducing sugars (mg/mL) | Total acids (%) | N-Amino (mg/mL) | pH     |
|----------------------------------|---------------------------|------------------|----------------------|-------------------------|----------------|----------------|--------|
| Yellow corn                      | 0.68                      | 92.98            | 11.09                | 6.68                    | Nd*            | 1.95           | -      |
| Nixtamalized yellow corn         | 0.19                      | 54.02            | 6.85                 | 1.27                    | 0.10           | 1.05           | 5.00   |
| Pulp of yellow corn nixtamalized** | 0.17                      | 17.83            | 37.20                | 27.00                   | 0.10           | 0.20           | 6.09   |
| White corn                       | 0.88                      | 90.95            | 7.06                 | 5.25                    | Nd*            | 1.80           | -      |
| Nixtamalized white corn          | 0.22                      | 53.10            | 4.53                 | 1.28                    | 0.10           | 1.20           | 5.54   |
| Pulp of white corn nixtamalized** | 0.20                      | 18.15            | 41.50                | 28.00                   | 0.10           | 0.18           | 6.08   |

Legend:*not detected according to titration method [7], **corn and water on a 1 : 4 ratio.

Nixtamalization process is performed through steeping corn with Ca(OH)2 solution and pulverizing in water on a 1 : 4 yielding pulp of corn nixtamalized. It is a turbid suspension, white and yellowish related with characteristic of initial material of corn. The whole processes of nixtamalization, composition of nixtamalized corn and pulp of corn nixtamalized from both types of corn are lower than that initial material of corn. Decreasing dissolved protein, N-Amino and total solids are possibility caused by its occurrence of denaturation of protein because of processes of blanching, pulverizing, and diluting. Meanwhile, total sugars and reducing sugars are get through pulverizing and diluting in hot water during nixtamalization process causing level of acidity drops or pH is higher. Total acids contained in corn, nixtamalized corn and pulp of corn nixtamalized tends to show similarity and not detected at initial material of corn. The presence of total acids is possibility caused by natural fermentation using Lactic Acid Bacteria (LAB) from steeping (18 hours). Figure 2a-2f demonstrate subsequent dry yellow corn, nixtamalized yellow corn, pulp of yellow corn nixtamalized, dry white corn, nixtamalized white corn, pulp of white corn nixtamalized using type of horse dent corn (Zea mays var. indenata).

Figure 2. (a) dry yellow corn, (b) yellow corn nixtamalized, (c) pulp of yellow corn nixtamalized, (d) dry white corn, (e) white corn nixtamalized, and (f) pulp of white corn nixtamalized.
3.2. Effect of fermentation process condition on composition of biomass

3.2.1. Dissolved protein (mg/mL) and N-Amino (mg/mL)

Both fermentations of nixtamalized yellow corn using inoculum of corn folate A and B, and nixtamalized white corn using inoculum of corn folate C and D are conducted with concentration of corn folate inoculum of 40% (w/v, dissolved protein) and 37 °C for 0, 8, 16 and 24 hours yield biomasses with dissolved protein and N-Amino, as indicated in Figures 3a and 3b. Dissolved protein and N-Amino are a parameter of the presence of folic acid before and after fermentation. Folic acid (L-glutamic acid, pteroyl-L-glutamic acid, Vitamin B<sub>9</sub>, Vitamin M, Folacin) (C<sub>19</sub>H<sub>19</sub>N<sub>7</sub>O<sub>6</sub>) is formed from a pteridine ring, a para-aminobenzoate (p-ABA) and a g-linked tail with one or more L-glutamates [12] so that the whole components indicate its presence of folic acid.

![Figure 3](a) (b)

**Figure 3.** Relationship between types of *Bifidobacterium* sp. inoculum, type of nixtamalized corn and fermentation time to recovery of (a) dissolved protein and (b) N-Amino of biomass of nixtamalized corn as source of natural folic acid.

Fermentation process shows a drop of dissolved protein and N-Amino with sufficient sharp between 0 and 8 hours. This matter is possibility caused by fermentation process experiencing adaptation phase of *Bifidobacterium* sp. although it had been enriched with skimmed milk (10%) and sucrose (10%) or *Bifidobacterium* sp. works very reactive. For 8, 16, and 24 hours of fermentation be visible metabolism activity of microbes. Recovery optimization of dissolved protein from nixtamalized yellow corn and nixtamalized white corn were achieved by biomass B (0.37 mg/mL) and biomass C (0.42 mg/mL) for 16 hours of fermentation being increasing dissolved protein in biomass B (117.64% or 1.18-folds) and biomass C (90.91%) compared to dissolved protein in initial material of both pulp yellow corn nixtamalized (0.17 mg/mL) and pulp of yellow corn nixtamalized (0.22 mg/mL). Meanwhile, recovery optimization of N-Amino was obtained by biomass A (0.27 mg/mL) and biomass C (0.26 mg/mL) for 16 hours of fermentation being increasing N-Amino of 35% and 44.44% compared to N-Amino in initial material of both pulp yellow corn nixtamalized (0.2 mg/mL) and initial material of both pulp nixtamalized white corn (0.18 mg/mL). Increasing both dissolved protein and N-Amino are not only possibility caused by contributions of skimmed milk and sucrose, but also by long fermentation time so that it is occurred fision of protein substrate due to proteolytic activity of *Bifidobacterium* sp. in degrading protein. Its formation of organic acids are also enabled to solve less part of protein as N-Amino during fermentation process. After optimum condition of fermentation time was achieved, further both these components decreased their concentrations to 24 hours of fermentation. This reason is possibility caused by its occurrence of lysis or denaturation by forming caramel compound being enabled by Maillard reaction because of interaction amongst fermentation temperature (37 °C), sucrose and protein substrates so that they are not detected by Lowry method as dissolved protein [6] or N-Amino [8].
3.2.2. Total sugars (mg/mL) and reducing sugars (mg/mL)

Total sugars in biomass is all remained sugars being used by microbes for their metabolisms after fermentation process. The presence of total sugars is affected by intrinsic factors of microbe (unproportional ratio of microbe and sucrose, microbe activity in fermenting sugars, environmental condition of fermentation). Whereas, reducing sugars is all type of sucrose (glucose, fructose, glycerol, galactose, lactose, maltose, etc.) being having an ability to reduce according to Somogyi Nelson method [7]. Reducing sugars is generated as a part of sucrose degradation as a result of enriching media or polysaccharides of nixtamalized corn by microbes. Total sugars and reducing sugars are of biomass are one of the important parameters of *Bifidobacterium* sp. activity in fermenting nixtamalized corn as source of carbon in order to form acids affecting on aroma and taste. Fermentation process indicates its occurrence of dropping sharply total sugars in both biomass A and biomass B, and increasing strongly total sugars in both biomass C and biomass D between 0 (initial) and 8 hours of fermentation. After 8 hours of fermentation, total sugars in both biomass C and biomass D decreases, however, it achieves optimization in both biomass A and biomass B, as demonstrated in Figure 4a. This reason showed that type of substrate factor, initial concentration of material prior to process and fermentation time affect on total sugars of biomass. Fermentation between 0 (initial) and 8 hours is enabled to adapt microbes and high activity of microbes in fermenting substrate so that after 8 hours of fermentation is occurred a delay of microbe activity because the whole energy for metabolism have been generated. Optimization condition of total sugars is obtained by both biomass B (104.74 mg/mL) for 16 hours of fermentation and biomass C (107.70 mg/mL) for 8 hours of fermentation. In this condition, it is occurred a increasing total sugars in biomass B (181.56% or 1.82-folds) and biomass C (159.52% or 1.59-folds) compared to both total sugars in initial concentration of material of pulp yellow corn nixtamalized (37.2 mg/mL) and pulp of white corn nixtamalized (41.5 mg/mL). At similar time, microbes conduct a degrading chemically in order to form metabolite (organic acids, etc.). Reducing sugars concentration increases in line with long fermentation time on the whole biomass, as shown in Figure 4b. Enriching sucrose and skimmed milk affected on activity of microbes so that stock of carbon source supports recovery of reducing sugars. Optimization of reducing sugars was achieved by biomass B (35.68 mg/mL) and biomass C (40.42 mg/mL) for 24 hours of fermentation. In this condition, it takes place an increase of reducing sugars in biomass B (32.15%) and biomass C (44.36%) compared to reducing sugars in initial material of pulp yellow corn nixtamalized (27 mg/mL) and pulp white corn nixtamalized 28 mg/mL).

![Figure 4](image1.png)

**Figure 4.** Relationship between types of *Bifidobacterium* sp. inoculum, type of nixtamalized corn and fermentation time to recovery of (a) total sugars and (b) reducing sugars of biomass of nixtamalized corn as source of natural folic acid.

3.2.3. Total acids (%), total solids (%) and level of acidity (pH)

Total acids is all organic acids as detected metabolite according to titration method [7] and is a parameter in metabolism process of homofermentative lactose [5] as activity of *Bifidobacterium* sp. Fermentation process yields a increase of total acids in line with long fermentation time being caused
by ability of Bifidobacterium sp. to degrade conversion of sugars to organic acids, particularly lactic acid, malic acid, etc. Bifidobacterium sp. will reduce pyruvic acid being formed through pathway of glycolysis by NADH2 to produce lactic acid [3]. Increasing total acid is also supported by stock of carbon source through enriching biomass by adding skim milk as sources of lactose and sucrose. Total acids in biomass A and biomass C is get higher compared to biomass B and biomass D, as displayed in Figure 5a. This reason indicated that activity of B. brevis is able to convert sugars to organic acids compared to B. bifidum. Optimization of total acids are obtained by biomass A (1.62%) and biomass C (1.06%) for 24 hours of fermentation. In this condition is occurred an increase of total acids of 1,520% (15.2-folds) and 960% (9.6-folds) compared to total acids in both initial material of pulp yellow corn nixtamalized (0.1%) and pulp of white corn nixtamalized (0.1%).

Rate of fermentation tends to yield total solids being fluctuating. Total solids are accumulation of the whole components both soluble and insoluble according to Gravimetric method [7]. Fermentation process generates optimization of total solids in biomass A for 16 hours (21.77%) being lower compared to total solids in biomass C for 8 hours (22.53%). In this condition, it is occurred an increase of total solids in biomass A (22.10%) and biomass C (24.13%) compared to total solids in initial material of pulp yellow corn nixtamalized (17.83%) and initial material of pulp white corn nixtamalized (18.15%). Difference in total solids from 4 types of biomass is not only possibility caused by total solids in initial material, but also by ability of Bifidobacterium sp. in fermenting nixtamalization, and contribution of enriched materials of skimmed milk and sucrose. In general, fermentation reaction of LAB, and ability to degrade component, particularly sugars and protein in yielding metabolites (organic acids and volatile compounds) are able to form and result substrate coagulation causing texture of biomass more dense, as shown at biomasses C and D between 8 and 24 hours of fermentation in Figure 5b.

**Figure 5.** Relationship between types of inoculum of corn folic acid of Bifidobacterium sp., type of nixtamalized corn and fermentation time to recovery of (a) total acids, (b) total solids and (c) pH of biomass of nixtamalized corn as source of natural folic acid.
On level of acidity (pH) is one of the main parameters in fermentation of LAB being related to total acids concentration as metabolite organic acids produced during fermentation process. Level of acidity (pH) of biomass is determined based on the presence of hydrogen ion (H+) related to total acid concentration of biomass. Period when organic acids concentration increase, level of acidity (pH) becomes more and more decrease. Interaction of treatment amongst type of nixtamalized corn, type of corn folate inoculum, and fermentation time generates linear drop of biomass pH in line with long fermentation time for the whole biomasses. This reason had showed that fermentation operates successfully, in which activity of Bifidobacterium sp. forming organic acids runs reactively. Figure 5c demonstrates that biomass A and C gives lower pH of suspension compared to biomass B and D.

This matter took place during fermentasi process in less neutral situation (pH 6). Pulping nixtamalized corn related to nixtamalization process using Ca(OH)2, which tends to base condition, so that it is sufficient reactive for the growth of Bifidobacterium sp. being reactive at optimum temperature of 37 – 41 °C, and optimum pH of 6.5 – 7 [13]. Based on type of nixtamalized corn, optimization of pH was achieved at biomass A and C yielding biomass pH of 4.08 and 4.38 for 24 hours of fermentation. In this condition, it dropped pH on biomass A (49.02%) and biomass C (28.08%) compared to pH of initial material of nixtamalized yellow corn porridge of 6.09 and pH of initial material of pulp white corn nixtamalized of 6.08 prior to ferment.

3.2.4. Optimum condition of fermentation

From evaluation mentioned above, it had been known that optimization time of fermentation process on nixtamalized corn based on the highest dissolved protein concentration as an indication folic acid was reached from biomass B and biomass C. In these conditions, biomass B and biomass C have compositions of folic acid of 213.58 µg/mL and 297.72 µg/mL, total solids of 20.60% and 21.01%, dissolved protein of 0.37 mg/mL and 0.42 mg/mL, reducing sugars of 34.54 mg/mL and 38.27 mg/mL, total sugars of 104.7 mg/mL and 98.6 mg/mL, total acid 0.75% and 0.88%, N-amino of 0.24 mg/g and 0.26 mg/g and pH 4.82 and 4.49. Figures 6a and 6b display biomass B and biomass C.

3.2.5. Identification of folic acid monomer and folic acid concentration

Analysis of standard folic acid is get 1 (one) peak (T 4.5) with retention time of 0 – 10 minutes and relative intensity of 100%, in which at mass spectra m/z 250 – 498 from T 4.5 show domination of monomer with molecular weight (MW) of 442.32, 443.60, and 442.93 Dalton (Da.) with relative intensities of 100, 41.63, and 27.5%, as displayed in Figures 7a and 7b.
Figure 7. (a) Chromatogram of standard folic acid, (b) mass spectra of standard folic acid, (c) chromatogram and (d) mass spectra of B, (e) chromatogram, and (f) mass spectra of biomass C at optimum condition of fermentation (37 °C, 16 hours).

It had been known that folic acid has MW of 441 Da. By means of LC-MS method had been known that a compound indicated by difference in MW, in which its possibility is as M+, M+ Na+, 2M++ or 2M+, Na+. Operation condition of LC-MS is injection volume of 5 μL, flow rate of 0.2 mL/minute with eluent of methanol and water on a 80 : 20 ratio, and C-8 (15 mm x 2 mm) column [9]. Analysis of identification on folic acid monomer is conducted in the best biomass, in which both biomasses B and C are nixtamalized yellow corn and nixtamalized white corn by using B. bifidum and B. brevis at 37 °C and concentration of 40% (w/v, dissolved protein) for 16 hours. Identification of folic acid monomer from biomasses B and C result chromatogram with 1 (one) peak as T 4.0 and T 3.8 with retention time of 1 – 10 minute, as shown in Figures 7c and 7e, which mass spectra of...
biomass gives 8 and 6 folic acid monomers dominated by folic acid with MW of 442.29 and 442.56 Da. and relative intensity of 100% and 100%, respectively, as indicated in Figures 7d and 7f.

### 3.2.6. Distribution of particle size

Biomass B and biomass C as a result of fermentation with inoculum concentration 40% at 37 °C for 16 hours is sufficient thick suspension with white and yellowish white in colour. Fermentation process generates texture of biomass as semi solids by activity of *B. bifidum* and *B. brevis* in fermentation of substrates. Type of materials, fermentation condition, and activities of *B. bifidum* and *B. brevis* are possibility affected by particle size and particle size distribution of biomass particle. Table 2 displayes smaller particle size of biomass B (1075.7 nm) compared to biomass C (1115.1 nm) and dispersed particle (Particle Index) of 0.827 an 0.849.

**Table 2.** Characteristic of biomass particle of nixtamalized yellow/white corn by inoculum of corn folate of *B. bifidum* and *B. brevis* at optimum condition of fermentation (37 °C, 16 hour).

| Types of biomass | Particle size distribution of nano folate (nm) | Z-Average (nm)* | PI** |
|------------------|-----------------------------------------------|-----------------|------|
| Biomass of nixtamalized yellow corn by inoculum of *B. bifidum* (B) | 1075.7 | 0.827 |
| Biomass of nixtamalized white corn by inoculum of *B. brevis* (C) | 1115.1 | 0.849 |

Legend : *Diameter of nano particles and **dispersed particles (Particle Index).

Difference in both particle size and particle size distribution are not only possibility caused by types of nixtamalized corn, but also by composition for the whole biomasses, particularly total solids. Biomass of B contains smaller total solids (20.6%) than that biomass of C (21.01%). Total solids are accumulation of the whole components both soluble and insoluble in water according to Gravimetric method [7] so that they have texture and smaller particle size. This difference in type of biomass appear at particle size distribution, in which biomass of B generate particles dispersion with diameter size (Ø) range of 120 – 240 nm (< 1000 nm) at dispersed particle (frequency) range of 0 – 25% and between 150 and 5500 (< 10000 nm), as indicated in Figure 8a.

**Figure 8.** Distribution of powder particles of (a) biomass B, (b) biomass C using inoculum of *B. bifidum* and *B. brevis* at optimum condition of fermentation (37 °C, 16 hour).

Biomass of C yield particles dispersion smaller, namely Ø of 120 – 200 nm (< 1000 nm) at dispersed particle (frequency) of 27.5%, and 120 – 7000 nm (< 10000 nm) at dispersed particle (frequency) of 17.5 – 35%, as demonstrated in Figure 8b. In analysis of particle size distribution according to DLS, particle size becoming more and more small will reduce particle index or in other words particle dispersion become more uniform and homogen. Both types of biomasses have particle index smaller than 1 (one) showing uniform particle size [14] so that it is easier to be adsorbed by digestive system.
Identification of volatile compound in biomass

Identification on volatile compound in biomass of B displayed chromatogram with 20 and 24 peaks dominated by volatile compound at the peaks 1, 2, 3, 6, 11, 17 and 20 subsequent as 2,3-butanediol, lactic acid, hexanoic acid, benzoic acid, dodecanoic acid, palmitic acid, and octadecenoic acid, whereas biomass of C demonstrated chromatogram with 20 and 24 peaks dominated by volatile compounds at peaks 3, 12, 13, 14, 22, 23, and 24 subsequent as 2,3-butanediol, palmitic acid, octadecenoic acid, stearic acid, palmitic acid, octadecenoic acid, and stearic acid. Biomass of B gave much more lactic acid (2.37%) compared to biomass C (0.53%), as shown in Figures 9a and 9b.

Lactic acid is organic compound produced through result of metabolite of B. bifidum, however, it is not found in biomass C. In the whole treatments, volatile compounds of biomasses of B and C was dominated by fatty acids originating from corn, particularly 2,3-butanediol of 4.46 and 10.65%, palmitic acid of 7.63 and 8.26%, octadecenoic acid of 6.31 and 9.5%, lactic acid of 2.37 and 0.53%, sugar compounds was only in biomass of D as glucopyranosiduronic acid (1.53%), benzoic acid (4.14%) was found in biomass of B as PABA derivatives, a part of compound from folic acid [12]. Biomass of C yielded much more volatile compound than that biomass of B.

4. Conclusion

The best fermentation time on both nixtamalized white corn and yellow corn were achieved for 16 hours by using inoculum B and inoculum C. They use B. bifidum and B. brevis, and give composition of folic acid of 213.58 µg/mL and 297.72 µg/mL, total solids of 20.60% and 21.01%, dissolved protein of 0.37 mg/mL and 0.42 mg/mL, reducing sugars of 34.54 mg/mL and 38.27 mg/mL, total sugars of 104.70 mg/mL and 98.60 mg/mL, total acids of 0.75% and 0.88%, N-amino 0.24 mg/g and 0.26 mg/g, and pH of 4.82 and 4.49. In this condition, it is occurred an increase of dissolved protein of 117.64% (1.18-folds) and 90.91%, total solids of 18.53% and 15.76%, reducing sugars of 27.92% and 36.68%, total sugars of 181.56% (1.8-folds) and 137.59% (1.37-folds), total acids of 649.4% (6.5-folds) and 780% (7.8-folds), N-Amino of 21% and 45.22%, and decrease of pH of 20.85% and 26.15%, respectively compared to compounds in nixtamalized white corn and nixtamalized yellow corn without fermentation being dominated by folic acid monomer with MW of 442.29 Da. and 442.56 Da., relative intensity of 100% with particle size of 1151.1 nm and 1075.7 nm, particle index of 0.827 and 0.849. Volatile compounds were dominated by 2,3-butanediol of 4.46% and 10.65%, palmitic acid of 7.63% and 8.26%, octadecenoic acid 6.31% and 9.5%, lactic acid of 2.37% and 0.53%.

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