Characteristic of Biomass of Corn (Zea Mays Identata) Fermented by Lactobacillus Acidophilus and Bifidobacterium Brevis as Source of Natural Folic Acid

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Abstract. Nixtamalized yellow and white corn from type of horse dent (Zea mays var. identata) fermented by Lactic Acid Bacteria (LAB) has potential use as source of natural folic acid. Fermentation uses four (4) types of corn inoculum expressed as inoculums A, B, C and D, i.e: Inoculum A (nixtamalized white corn, sucrose 10% w/w, skim milk 10% w/w using inoculum L. acidophilus 30% fermented 8 hours), inoculum B (nixtamalized white corn without sucrose and skim milk using inoculum Bif. brevis 30% fermented 24 hours), Inoculum C (nixtamalized yellow corn, sucrose 10% w/w, skim milk 10% w/w with inoculum L. acidophilus 30% fermented 16 hours), and inoculum D (nixtamalized yellow corn, sucrose 10% w/w, skim milk 10% w/w with inoculum Bif. brevis 30% fermented 8 hours). Fermentation was done at inoculum concentrations 0, 10, 20, 30, and 40 % (v/w dissolved protein) and 37 °C for 24 hours. Result of experiment work showed that optimisation fermentation were reached by biomass B and biomass D at inoculum concentration 40 % with composition of folic acid 103.07 and 91.92 µg/mL, followed by particle sizes 987.4 and 762.8 nm, folic acid monomer with (MW) 443.70 and 442.96 (Da).

1. Introduction
Corn (Zea mays L.) is one of the global cereal grains crops of multiple uses as source of polysaccharides for energy and body builder, and sufficient high source of protein (± 9 %) [1]. High protein content emergences also corn as potential source of folic acid besides other bioactive compounds (β-carotene, etc.). Folic acid or folate (natural form) is an very essential micro-nutrient in the B-complex vitamins for physiological processes, particularly growth and fetal development from synthesis of nucleotideto remethylation of homocysteine, contributing to the formation, fission and growth of (new) cells and tissues. Infants and human adult need folic acid to produce red blood cells and prevent anemia [2]. Folic acid has role as co-enzyme synthesis of methionine, purin and pyrimidine. Specific function of co-enzyme of folate in body is as a mediator in transferring units of single carbon. Co-enzyme of folate has central role as acceptor and donor from one-carbon donation in various reactions to metabolize nucleic acid and amino acids. Folic acid (folate, folacin, vitamin B9, pteroyl-L-glutamic acid, pteroyl-L-glutamate, pteroyl monoglutamic acid) with chemical structure, as shown in figure 1 [3].
It had been known that folic acid consist of a pteridine ring originating from 6-hydroxymethyl-7,8-dihydropterin pyrophosphate (DHPPP), bound to para-aminobenzoic acid (pABA) produced by plants [4], and is precursor in forming folic acid. Biosynthesis of DHPPP is occurred through four (4)steps covering formation of pterin ring structure by Amadori rearrangement, defosforilation pterin to aldolase, which will actify DHPPP into (7,8-Dehydropteroate (DHP) followed by synthase enzyme of dehydrofolate (EC-6.3.2.12) to dehydrofolate (DHF), and reducing by reductase enzyme of DHF (EC 1.3.5) to co-factor of tetrahydrofolate (THF) followed by THF-polyglutamate with adding glutamatey enzyme of folypolyglutamate synthase (EC 6.3.2.17) [3]. LAB produces also other metabolites, such as organic acids particularly lactic acid by enzymatic activity of β-D-Galactosidase followed by a small number of CO₂, taste and specific aroma like yoghurt [5]. Soluble carbohydrate of corn is source of carbon on the growth of LAB, and sucrose and skimmed milk. Modification treatment of nixtamalization using lime solution Ca(OH)₂ increases folic acid content by its occurrence of binding and adsorbing of Ca(OH)₂ [6], however it differs with nixtamalization process in general. Nixtamalized corn is as substrate for the growth of LAB, in which L. acidophillus and B. brevis besides others LAB will not only degrade sucrose to organic acids, particularly lactic acid, but also it is as agent which is able to synthesize folic acid through de Novo biosynthesis. Fermentation of LAB by inoculums of L. acidophillus and B. brevis with and without both enriching skimmed milk and sucrose on different substrate of nixtamalized corn enable to be known optimization of recovery folic acid as source of natural folic acid.

In order to find out characteristic of folic acid monomer produced by fermentation process, their identifications through Liquid Chromatography coupled with Mass Spectrometry (LC-MS) enables to be known folic acid monomer domination of biomass. By means of chromatography will be separated mixture of molecule based on difference in migration speed and distribution of molecules in stationary phase (adsorben) and mobile phase (eluen), meanwhile mass spectrometry will ionize analyt based on principle of electro spray ionisation (ESI) to gas phase (fine aerosol) [7]. LC-MS will separate folic acid monomer and identify based on molecular weight (MW) and relative intensity. Analysis of particle size distribution is conducted by means of LS instrument, in which diffraction of laser beam with particles as main source of particle size information. Its principle is laser radiation (λ 750 nm) pass through filter spatial and projection lens to form light group (beam). Then, it passes cell sample, in which suspended particles in liquid diffusent light toward object in certain characteristic according to appropriate particle size. Model characteristic measured is amount of diffraction for each represented particle size in samples. Distribution of particle size is calculated according to Fraunhofer or Mie method from measurement result of 126 detectors placed with angle 35° from optical axis [8, 9, 10]. This analysis is performed to find out size and distribution, in which particle size in biomass becoming more and more small will ease adsorption of folic acid on digestive system.

The goal of experiment work was to find out the best fermentation process condition through difference in types of nixtamalized corn from variety of horse dent, type and concentration of LAB inoculum (L. acidophilus and B. brevis), with and without both enriched sucrose and skimmed milk on composition of the whole biomasses, particularly folic acid, type of dominant folic acid monomer, and distribution of particle size.
2. Materials and Methods

2.1 Materials and Equipments
Main materials used in this experimental work were dry yellow corn and dry white corn (*Zea mays* var. *indentata*) procured from a local market, culture of *Bifidobacterium brevis* and *Lactobacillus acidophilus* (FNCC-UGM), MRS B (Man-Rogosa-Sharpe Broth, Merck), Ca(OH)$_2$ (E.Merck), standard folic acid (E.Merck), skimmed milk (Benato, USA), sucrose (local), distilled water, and chemicals. Chemicals used in this process and analysis were standard folic acid (Aldrich), 3-aminophenol (Aldrich), hydrochloric acid (E.Merck), sodium nitrite (E.Merck), and sulphamic acid (E.Merck). All the chemicals used in this process and analysis were reagent grade, procured locally, and used without further purification. Main equipments utilized in this experimental activity were Balance (Fujitsu, Japan), series of nixtamalization process system in laboratory scale, pan, blender (National, local), and equipments for microbiology process (autoclave, laminar air flow, incubator). Main instruments for analysis were UV-vis Spectrophotometer (Model RF-550, Shimadzu, Japan), and Liquid Chromatography coupled with Mass Spectrometer (LC-MS) (Mariner Biospectrometry) with LC (Hitachi L 6200) and Particle Size Analyzer (PSA) by PSA Coulter SZ 100 (Horiba Nano Partica).

2.2. Experimental design
Experimental activity was conducted through fermentation process on substrates of nixtamalized white corn by inoculums A and B, and nixstamalized yellow corn by inoculums of C and D at concentrations of 0, 10, 20, 30 and 40 % (v/w dissolved protein) and 37 °C for 24 hours, respectively so that it was yielded biomasses of A, B, C and D. Analysis was carried out dissolved protein (Lowry method) \[11\], total solids (Gravimetric method), reducing sugars (Somogyi-Nelson) \[12\], total sugars (Phenol Sulphate method) \[13\] and folic acid (spectrophotometri UV-vis) \[14\]. Identification on folic acid monomer was done by means of LC-MS (Mariner Biospectrometry) with LC (Hitachi L 6200) \[7\], and distribution of particles was measured by Particle Size Analyzer (PSA) [PSA Coulter SZ 100 (Horiba Nano Partica)] \[15\]. Process and analysis were conducted in duplicate. Data processed in this description were based on the result of average analysis.

2.3. Procedure

2.3.1 Nixtamalization of corn
A number of yellow corn and white corn from variety types of horse dent (*Zea mays* var. *indentata*) was cleaned, separated from dirt, washed and steeped in water at corn and water ratio of 1 part : 4 parts by adding lime solution [Ca(OH)$_2$] with concentrations of 20 % (yellow corn) and 30 % (white corn) for 18 hours respectively \[16\]. Further, it was blanching for 60 minutes (yellow corn) and 30 minutes (white corn), cooled at room temperature and pulverized by blender at full speed until nixtamalized corn pulp was produced.

2.3.2. Preparation of cultures stock of *L. Acidophilus*, *B. bifidum* and corn-folic inoculum
A number of MRS B media (15.6 g) was weighed, added aquadest 300 mL autoclaved at 121 °C for 15 minutes, cooled and incubated by starter of *L. acidophilus* and *B. bifidum* 1 % (w/v MRS B), and incubated at 37 °C for 24 – 36 hours until the growth of microbes in media had been seen. Preparation of corn folate inoculum was performed by mixing one (1) part of nixtamalized yellow corn pulp and two (2) parts of water, respectively. Inoculum A was prepared from mixing nixtamalized white corn pulp, sucrose (10 % w/w) and skimmed milk (10 % w/w), autoclaving at 121°C for 15 minutes, cooling at room temperature, inoculating by culture of *L. acidophilus* 30 % (v/w dissolved protein of nixtamalized corn), and fermentating at 37 °C for 8 hours. Inoculum B was prepared from mixing nixtamalized white corn pulp, autoclaving at 121°C for 15 minutes, cooling at room temperature, inoculating by culture of *B. brevis* 30 % (v/w dissolved protein of nixtamalized corn), and fermentating at 37 °C for 24 hours. Inoculum C was prepared from mixing nixtamalized yellow corn pulp, sucrose
(10 % w/w) and skimmed milk (10 % w/w), autoclaving at 121°C for 15 minutes, cooling at room temperature, inoculating by culture of *L. acidophilus* 30 % (v/w dissolved protein of nixtamalized corn) and fermenting at 37 °C for 16 hours. Meanwhile, inoculum D was prepared from mixing nixtamalized yellow corn pulp, sucrose (10 % w/w) and skimmed milk (10 % w/w), autoclaving at 121 °C for 15 minutes, cooling at room temperature, inoculating by culture of *B. brevis* 30 % (v/w dissolved protein of nixtamalized corn) and fermenting at 37 °C for 8 hours [16].

### 2.3.3. Fermentation of LAB

A number of nixtamalized white corn pulp (400 mL) and nixtamalized yellow corn pulp (400 mL) was added by sucrose (10 % w/w) and skimmed milk (10 % w/w), autoclaved at 121 °C for 15 minutes, cooled at room temperature, inoculated by corn inoculum A on nixtamalized white corn, and inoculums C and D on nixtamalized yellow corn 10, 20, 30 and 40 % (v/w dissolved protein) and incubated at 37 °C for 24 hours until fermented corn biomasses A, C and D were produced, respectively. Another treatment, a number of nixtamalized white corn pulp (400 mL) was autoclaved at 121 °C for 15 minutes, cooled at room temperature, inoculated by inoculum B with concentrations of 10, 20, 30 and 40 % (v/w dissolved protein), and incubated at 37 °C for 24 hours, respectively until fermented corn biomass of B was produced.

### 3. Results and Discussions

#### 3.1. Characteristics of materials

Compositions of white corn and yellow corn showed difference in both, as summarized in Table 1. White corn indicated both higher concentrations of folic acid (2.11 µg/mL) and total solids (86.35 %) comparable to folic acid (2.11 µg/mL) and total solids (77.79 %) in yellow corn. Difference in this composition is possibility caused by difference in type of variety and treatment of post harvesting.

| Kind of material | Compositions | Folic acid (µg/mL) | Total solid (%) | Reducing sugar (mg/mL) | Total sugar (mg/mL) | Dissolved protein (mg/mL) | Total acid (%) |
|------------------|--------------|--------------------|-----------------|------------------------|---------------------|--------------------------|----------------|
| White corn       |              | 2.12               | 86.35           | 42.82                  | 18.90               | 1.07                     | n.d.           |
| Yellow corn      |              | 2.11               | 77.79           | 26.38                  | 37.80               | 1.20                     | n.d.           |
| Nixtamalized white corn** |            | 16.94               | 45.68           | 12.56                  | 86.00               | 0.62                     | 0.0976         |
| Nixtamalized yellow corn** |          | 17.24               | 64.00           | 12.45                  | 98.00               | 0.33                     | 0.0979         |

Legend : *not detected (n.d.) according to titratable method [17], ** as suspension/pulp.

A series of nixtamalization process of corn conducted by steeping dried corn with lime solution [Ca(OH)₂] and pulverizing at corn and water ratio of 1 part and 4 parts produces nixtamalized corn pulp, which is turbid suspension, with color and yellowish relating with initial characteristic of corn material. The whole compositions experiences a change, in which both folic acid and total sugars higher, however total solids, dissolved protein and reducing sugars lower comparable to initial material of corn. Increasing folic acid is possibility caused by its occurrence of binding components by Ca(OH)₂, whereas total sugars is caused by process of pulverizing and diluting in water by heat. Decreasing dissolved protein and total solids are possibility caused by its occurrence of protein denaturation due to blanching, pulverizing and diluting at corn and water ratio of 1 : 4. On total acids, both type of nixtamalized corn tend not to show in difference. Its presence of total acids are enabled its occurrence of natural fermentation by LAB for 18 hours of steeping. Figures 2 (a), (b), (c) and (d) showed subsequently white corn, nixtamalized white corn pulp, yellow corn, and nixtamalized yellow corn pulp from variety type of horse dent.
Figure 2. (a) white corn, (b) nixtamalized white corn pulp, (c) yellow corn, and (d) nixtamalized yellow corn pulp from variety of horse dent.

3.2. Effect of fermentation process condition on composition of biomass

3.2.1. Folic acid (µg/mL)
Fermentation on nixtamalized white corn by inoculum A and B, and nixtamalized yellow corn by inoculum C and D at concentrations 0, 10, 20, 30 and 40 % (w/v dissolved protein) and 37 °C for 24 hours yields biomass with folic acid at specific optimum condition for each LAB. Folic acid at biomass of white corn with inoculum B shows fluctuative pattern and the best folic acid is achieved at concentration of inoculum 40 % (103.07 µg/mL) higher comparable to inoculum A and the optimum folic acid is achieved at concentration of inoculum 20 % (61.44 µg/mL). Both results display fluctuative rate of fermentation on folic acid, as seen in figure 3 (a). Trend on almost same rate of fermentation seems afermentation of nixtamalized yellow corn, in which both inoculum C and inoculum D generate more linear increase of folic acid. Biomass C results the best folic acid at concentration of inoculum 20 % (63.87 µg/mL), meanwhile biomass D produces folic acid 91.92 µg/mL at concentration of inoculum 40 %, as demonstrated in figure 3 (b). Difference in optimization on four (4) types of these inoculum are not only caused by different specification of inoculum, but also it is possibility caused by activities from four (4) LABs in fermentation of substrate. Biomasses A, C and D are produced by inoculum A, C, and D with enhancing skimmed milk and sucrose, respectively. This enhancing contributes on nutrition of LAB more activated in forming lactic acid and other organic acids as a result of converting piruvate acid through glycolysis pathway (EMP) [18]. Formation of high organic acids enable to be its occurrence degradation of substrate protein so that it is occurred lysis and inhibit formation of folic acid.

Figure 3. Relationship between type and concentration of LAB inoculum on recovery of folic acid in both nixtamalized yellow and white corn.

On biomass B, which uses without enhancing skimmed milk and sucrose in nixtamalized corn causes reaction based on source of carbon, so that formation of folic acid is concentrated and it becoming more and more increase to the highest concentration of inoculum D (40 %). Forming folic acid from probiotic bacteria is conducted through pathway of biosynthesis de novo, in which 6-hydroxymethyl-7,8-dihydropterin pyrophosphate (DHPP) linked to para-aminobenzoate (pABA) produced by plants and bacteria via pathway of pentose phosphate [3]. Activity of LABs have possibility key role on this
fermentation, in which *L. acidophilus* has the best growth condition at 35 - 38°C range and pH 5.5 – 6.0, while the growth of *B. brevis* is at 25 – 41°C range and is bad at pH > 8.0 or pH < 4.5 so that it has tolerance better comparable to *L. acidophilus*. From the whole fermentation processes, it had been known that the optimum condition in recovering folic acid on biomass of both nixtamalized white corn and yellow corn were achieved by inoculum B and inoculum D at concentration 40 % (w/v dissolved protein) with folic acid 103.07 and 91.92 µg/mL, respectively. In this condition, it is yielded increase of both folic acid in biomass of fermented white corn 508.44 % (5 times) and in biomass of fermented yellow corn 433.18 % (4.33 times) compared with folic acid in nixtamalized white corn without fermentation (16.97 µg/m) and nixtamalized yellow corn without fermentation (17.24 µg/m).

### 3.2.2. Dissolved protein (mg/mL)

Dissolved protein is a parameter on its presence of folic acid and other amino acids, before and after fermentation process. Interaction amongst treatment of nixtamalized corn type, and type and concentration of inoculum displays increase of dissolved protein followed by drop of dissolved protein at biomass of nixtamalized white corn for the whole treatments of inoculum A, however it increase at treatment of inoculum B, as showed in figure 4.

![Graph showing the relationship between type and concentration of LAB inoculum on recovery of dissolved protein in both nixtamalized yellow and white corn.](image)

**Figure 4.** Relationship between type and concentration of LAB inoculum on recovery of dissolved protein in both nixtamalized yellow and white corn.

Fermentation by inoculum A and B give the best concentration of dissolved protein at concentration of inoculum 10 % (0.45 mg/mL) and 40 % (0.15 mg/mL). This difference in dissolved protein concentration is possibility caused by biomass A by adding enhancing skimmed milk and sucrose so that lower concentration of inoculum (10 %) is resulted dissolved protein higher comparable to biomass B needing higher concentration of inoculum (40 %) to yield higher dissolved protein. In this condition, fermentation by inoculum A and B drops dissolved protein of biomass 25 % and 74.19 %, respectively comparable to dissolved protein in both nixtamalized white corn and yellow corn without fermentation (0.62 and 0.33 mg/mL). Fermentation process on nixtamalized yellow corn by inoculum C and D show dropping dissolved protein because of increase of inoculum concentration. The best treatment is reached at initial inoculum concentration (0 %) or without adding inoculum giving dissolved protein are 0.24 and 0.76 mg/mL, respectively. Comparable to dissolved protein in nixtamalized yellow corn without fermentation, fermentation by inoculum C and D increases dissolved protein 27.27 % and 130 % (1.3 times) comparable to dissolved protein in nixtamalized yellow corn without fermentation of LAB (0.33 mg/mL). This increase is naturally caused by its occurrence of microbes in nixtamalization process or hydrolysis of protein in nixtamalized corn by accumulation of heat (37 °C) during fermentation process.

### 3.2.3. Reducing sugar (mg/mL)
Reducing ability of reducing sugars (glucose, fructose, glyceraldehyde, galactose, lactose, maltose, etc.) according to Somogyi-Nelson method [12] on biomass of fermentation result is a parameter on activity of *L. acidophilus* and *B. brevis*. Interaction amongst treatment of nixtamalized corn type, type of inoculum and concentration of inoculum shows decrease of reducing sugars for the whole treatments on biomass of nixtamalized white corn by inoculum A, however it increase on treatment of inoculum B. Biomass of white corn fermented by inoculum A gives reducing sugars higher for the whole treatments comparable to using inoculum B, and the best conditions are achieved by inoculum A at 0 % (61.28 mg/mL) and inoculum B at concentration 40 % (23.37 mg/mL). Different trend seems at biomass of nixtamalized yellow corn, in which decrease of reducing sugars is occurred at the whole treatments both using inoculum C and inoculum D. Inoculum D gives reducing sugars higher for the whole treatments comparable to using inoculum C. The best fermentation condition on recovery of reducing sugars is achieved by inoculum C and D at concentration 0 % or without adding inoculum 49.11 mg/mL and 63.71 mg/mL, respectively, as demonstrated in figure 5.

![Figure 5. Relationship between type and concentration of LAB inoculum on recovery of reducing sugars in both nixtamalized yellow and white corn.](image)

This difference in result of reducing sugars had been known that activity of different LAB is effected by fermentation condition factors, particularly type of substrate, and type of LAB, and concentration of initial materials. Reducing sugars with lower molecular weight will be easier to be adsorbed by *L.* and *B. brevis* in its metabolisms. This matter causes adsorption on higher reducing sugars so that concentration of remained reducing sugars in biomass is lower [19]. It had been seemed that *L. acidophilus* as inoculum A and C is able to use the whole carbon sources from substrates to metabolize so that by it is becoming more and more increase of concentration of inoculum becomes less remained reducing sugars in biomass. Reducing sugars detected is a part of remained carbon source utilized for its metabolisms during fermentation. This matter seems at treatment without adding inoculum (0 %), in which higher remained reducing sugars compared with the whole treatments. Inoculum B in biomass of nixtamalized white corn and inoculum D in biomass of nixtamalized yellow corn with and without adding skimmed milk and sucrose remains reducing sugars becoming more and more high relating with increasing concentration of inoculum B, meanwhile by using inoculum D will recover reducing sugars becoming more and more drop relating with increase of inoculum concentration. From the whole assessments, it had been known that the optimum condition in recovering reducing sugars in biomass of both nixtamalized white corn and yellow corn are reached by inoculum A and inoculum D at concentration 0 % (w/v dissolved protein) and give reducing sugars 61.28 and 63.71 mg/mL. In this condition, it is yielded increase of reducing sugars in biomass of fermented white corn 387.9 % (3.9 times) and biomass of fermented yellow corn 411.73 % (4.1 times) compared with reducing sugars in nixtamalized white corn without fermentation (86 mg/mL) and nixtamalized yellow corn without fermentation (12.45 µg/mL).
3.2.4. Total sugar (mg/mL)

Total sugars in biomass of nixtamalized corn by using inoculum A and inoculum B is all remained sugars after fermentation process and a parameter of LAB in fermentation. Enhancing skimmed milk and sucrose contributes possibility on total sugars, in which fermentation by inoculum A and C gives total sugars higher comparable to by inoculum B and D. Fermentation on nixtamalized white corn by inoculum A and B are achieved at concentrations 10 % (154.79 mg/mL) and 40 % (118.4 mg/mL). Fermentation on biomass of nixtamalized yellow corn by inoculum C and inoculum D displays an increase of total sugars higher than total sugars in fermentation D with fluctuated rate of fermentation, as seen in figure 6. The best condition of fermentation is achieved at concentrations 40 % (216.33 mg/mL) and 10 % (w/v dissolved protein) (120.33 mg/mL). This difference in optimization on both types of nixtamalized corn is caused by enhancing skimmed milk and sucrose during fermentation by inoculum A, C and D, and without enhancing skimmed milk and sucrose for inoculum B. This matter causes remained total sugars in biomass of inoculum A, C and D higher comparable to biomass by inoculum B. This condition is possibility caused by sufficient saturated LAB due to nutrition, so that not all nutrients are able to be used, although LAB concentration increases, as well. Inoculum B seems to have ability more to use substrate in order to metabolize because by it is becoming more and more high inoculum concentration, remained total sugars is becoming more and more low relating with increasing inoculum concentration.

![Figure 6. Relationship between type and concentration of LAB inoculum on recovery of total sugars in both nixtamalized yellow and white corn.](image)

From the whole treatments, it had been known that the optimum condition in recovering total sugars at biomass of both nixtamalized white corn and yellow corn are achieved by inoculum A and inoculum C at concentrations 10 % (216.33 mg/mL) and 40 % (w/v dissolved protein) (154.79 mg/mL). In this condition, it is resulted an increase of total sugars in both biomass of nixtamalized white corn 151.55 % (1.5 times) and yellow corn sebesar 71.66 % (0.7 times) with comparable to initial total sugars in both nixtamalized white corn (86 mg/mL) and nixtamalized yellow corn (96 mg/mL).

3.2.5. Total solid (%)

Total solids is accumulation on the whole components both soluble and insoluble according to Gravimetric method [13]. Fermentation process on both nixtamalized white corn and yellow corn by inoculum L. acidophilus (A and C) and B. brevis (B and D) at concentration becoming more and more increase yields biomass with different total solids. Enhancing or without enhancing skimmed milk and sucrose in substrate and LAB inoculum contribute on recovery of total solids of biomass. Fermentation process on biomass of nixtamalized white corn by inoculum A and inoculum B indicates linear rate of fermentation and tends to drop and it is becoming more and more increase inoculum concentration for
the whole treatments. Fermentation process by inoculum A gives total solids higher comparable to by inoculum B due to enhancing skimmed milk and sucrose at biomass A. Based on the best fermentation condition, total solids in biomass of nixtamalized white corn by inoculum A and inoculum B is reached at concentration 0 % (23.43 %) or without inoculum (8.08 %). Rate of fermentation on total solids in biomass of nixtamalized yellow corn by inoculum C and D seems to be fluctuative, however tends to decline, in which total solids produced by inoculum D is higher comparable to inoculum B, as seen in figure 7. The best fermentation condition on biomass of nixtamalized yellow corn by inoculum C and D is achieved at concentrations 10 % and 40 % (w/v dissolved protein) and gives recovery of total solids 20.82 % and 20.97 %, respectively. Difference in optimization of total solids on four (4) biomasses are possibility caused by initial total solid of material, LAB ability to ferment nixtamalized corn and the best condition of LAB fermentation. As reaction of LAB fermentation in general, ability to degrade components, particularly sugars and protein in producing metabolite (organic acids and volatile compounds) is able to cause its occurrence of substrate coagulation, so that it is formed coagulant followed by agglomerating substrates. This matter causes biomass texture to more solid, however total solids is accumulation on all components both soluble and insoluble [20], so that it decreases the whole total solids. From the whole assessments, it had been known that the optimum condition in recovering total solids on biomasses of both nixtamalized white corn and yellow corn are achieved by inoculum A and inoculum D at concentration 0 % and 40 % (w/v dissolved protein) and give total solids 23.43 % and 20.97 %, respectively. In this condition, it is occurred a decline of total solids in biomasses of both nixtamalized white corn and yellow corn 48.7 % and 67.23 % higher comparable to initial total solids of nixtamalized white corn (45.68 %) and initial total solids of nixtamalized yellow corn (64.00 %).

![Figure 7. Relationship between type and concentration of LAB inoculum on recovery of total solids in both nixtamalized yellow and white corn.](image)

3.2.6. **Total Acid (%)**

Total acids titratable is all organic acids, particularly lactic acid, maleic acid, acetic acid, etc. detected according to titrimetri method [17] as metabolite and is parameter from its occurrence of hom fermentative lactose metabolisms [21], and other sugars as biochemistry activity of *L. acidophilus* and *B. brevis* utilizing source of carbon. Enhancing biomass by adding skimmed milk as source of lactose and sucrose contribute and increase total acids. Fermentation process on nixtamalized white corn by inoculum A increase total acids, whereas by inoculum B fluctuates for all treatments. Total acids as a result of fermentation by inoculum A higher compared with inoculum B because of enhancing skimmed milk and sucrose at biomass A. The best condition of fermentation based on total acids in biomass of nixtamalized white corn by inoculum A and B are achieved at concentration 40 % and 20 % with total acids 0.41 % and 0.17 %. This matter shows that enhancing skimmed milk and sucrose causes ability of *L. acidophilus* to increase total acids to the highest concentration (40 %), while ability of *B. brevis* without enhancing skimmed milk and sucrose is only at 20 %. Fermentation process on nixtamalized...
yellow corn by inoculum C and D indicates increasing total acids at concentration 20 % and the best total acids are at concentration 30 % and give total acids 0.29 %. This condition is occurred by adaptation phase of LAB processing slow by various factors or level of LAB saturated to stagnant phase followed by dropping total acids at concentration 40 %, as showed in figure 8. From the whole assessments, it had been known that the optimum condition in recovering total acids at biomass of nixtamalized white corn is achieved by inoculum A at concentration 40 % with total acids 0.41 %, meanwhile the optimum condition in recovering total acids based on efficiency on biomass of nixtamalized yellow corn is reached by inoculum C at concentration 20 % (w/v dissolved protein) with total acids 0.29 %. In this condition, it is occurred an increase of total acids in both biomasses of nixtamalized white corn 320.08 % (3.2 times) and nixtamalized yellow corn 196.22 % (1.9 times) comparable to initial total acids in nixtamalized white corn (0.0976 %) and initial total acids in nixtamalized yellow corn (0.0979 %).

Figure 8. Relationship between type and concentration of LAB inoculum on recovery of total acids in both nixtamalized yellow and white corn.

From the whole assessment, it had been known that based on the highest folic acid concentration, optimization of fermentation process on nixtamalized corn is achieved at nixtamalized white corn and nixtamalized yellow corn by inoculum B (B. brevis, without enhancing skimmed milk and sucrose) and inoculum D (B. brevis, with enhancing skimmed milk and sucrose at concentration 40 % (w/v dissolved protein). In this condition, biomass of nixtamalized white corn and biomass of nixtamalized yellow corn have compositions of folic acid 103.07 µg/mL and 91.92 µg/mL, dissolved protein 0.15 mg/mL and 0.20 mg/mL, total sugars 70.27 mg/mL and 92.95 mg/mL, reducing sugars 23.37 mg/mL and 26.94 mg/mL, and total solids 7.53 % and 20.97 %, respectively. In this condition, it is occurred an increase of folic acid 508.44 % (5 times) and 433.18 % (4.33 times), and reducing sugars 86.07 % (0.86 times) and 116.38 % (1.16 times), however it is occurred a decline of dissolved protein 75.81 % and 45.45 %, total sugars 18.29 % and 5.15 %, and total solids 83.51 % and 67.23 % comparable to components on nixtamalized white corn and nixtamalized yellow corn without fermentation. Figures 9 (a), (b), (c) and (d) indicates subsequently inoculum B, biomass B, inoculum D, and biomass D as a result of fermentation process at the optimum condition of fermentation process.
Figure 9. (a) Inoculum B, (b) biomass B at concentration of inoculum B 40 % (w/v dissolved protein) and 37 °C for 24 hours, (c) inoculum D, and (d) biomass D at concentration of inoculum D 40 % (w/v dissolved protein) and 37 °C for 24 hours.

3.2.7. Identification of folic acid monomer
Analysis on standard folic acid is yielded one (1) peak (T1.7) with retention time 0 – 10 minutes and relative intensity 100 %, in which mass spectra m/z 250 - 498 from T1.7 demonstrates monomer domination of molecule weight (MW) 442.76, 443.70 and 441.48 Dalton (Da.) with relative intensity 100 %, 25 %, and 5 %, as demonstrated in figures 10 (a) and (b). Folic acid has MW 441 Da. By means of LC-MS method had been known that a compound indicated difference in MW, in which its possibility is as M⁺, M⁺ Na⁺, 2M⁺⁺ or 2M⁺⁺ Na⁺. This matter is caused by its presence of ionization as a consequence of sensitivity of LC-MS instrument relating to eluent used. This matter caused by ionization because sensitivity of LC-MS instrument relating to eluent used. Operation condition of LC-MS is injection volume 5 μL and flow rate 0.2 mL/minute with eluent mixture of methanol and water at ratio 80 : 20, by using column C-8 (15 mm x 2 mm) [7]. Analysis identified on folic acid monomer is performed on the best biomass, namely biomass B and D with B. brevis, with and without enhancing skimmed milk and sucrose at concentration 40 % (w/v dissolved protein) and 37 °C for 24 hours. Idensification on folic acid monomer in biomasses B and D generate chromatogram with one (1) peak as T2.2 and T2.0 with retention time 01 -10 minute, as shown in figures 10 (c) and (e), in which mass spectra of biomass yields 6 and 8 folic acid monomer dominated by folic acid with MW 442.14 Da. and 442.96 Da. and relative intensity 90 % and 100 %, as displayed in figures 10 (d) and (f). Table 2 shows characteristic of folic acid monomer from two (2) biomasses.

Figure 10. (a) Chromatogram on standard folic acid, (b) mass spectra on standard folic acid, (c) chromatogram on biomass B, (d) mass spectra on biomass B, (e) chromatogram on biomass D, and (f) mass spectra on biomass D.

Table 2. Monomer dominant as folic acid on mass spectra extract of Biomass of white corn nixtamal using inoculum B and biomass of yellow corn nixtamal using inoculum D.

| Index | Centroid Mass | Relative Intensity (%) | Area | Index | Centroid Mass | Relative Intensity (%) | Area |
|-------|---------------|------------------------|------|-------|---------------|------------------------|------|
|       |               |                        |      |       |               |                        |      |
3.2.8. Distribution of particles

Biomass of nixtamalized white corn and biomass of nixtamalized yellow corn fermented by inoculum B and inoculum D, which is sufficient thick suspension with white to yellowish white color as a result of fermentation at inoculum concentration 40 % and 37 °C for 24 hours. Fermentation process generates biomass texture as a result of coagulant by LAB activity in fermenting substrate. Type of material, fermentation condition factor, and type of LAB affect on size and distribution of particles of biomass. Table 3 shows distribution of particles both type of biomasses, in which biomass of nixtamalized white corn by inoculum B resuls larger particles size (987.4 nm) with higher dispersed particles (Particle Index) (2,384) comparable to biomass of nixtamalized yellow corn by inoculum D 762.8 nm with dispersed particle (Particle Index) sebesar 2,085.

Table 3. Characteristic of folic acid particle at biomass of white corn and yellow corn as a result of fermentation by inoculum B and D.

| Type of biomass          | Type of LAB inoculum                     | Particle distribution of nano folic (nm) |
|-------------------------|-----------------------------------------|----------------------------------------|
|                         |                                         | Z-Average (nm)* | PI**        |
| Nixtamalized white corn | B. brevis without enhancing skinned milk and sucrose (B) | 987.4          | 2.384      |
| Nixtamalized yellow corn| B. brevis with enhancing skinned milk and sucrose (D) | 762.8          | 2.085      |

Legend : *Diameter of nano particle;**dispersed particles.

Difference in this result is caused by biomass of nixtamal white corn by inoculum B without enhancing skinned milk and sucrose so that corn granula will dominate suspension, meanwhile biomass of nixtamalized yellow corn by inoculum D with enhancing skinned milk and sucrose so that texture and particles size are smooth and uniform. This matter is also known from difference in total solids, in which biomass affects on nixtamalized white corn having lower total solids (7.53%) compared with biomass of nixtamalized yellow corn 20.97 %, so that this influences on dispersed particles (PI) of biomass. This difference seems on distribution of particles from biomass of nixtamalized white corn by inoculum B and biomass of nixtamalized yellow corn by inoculum D, in which it produces particles with diameter size (Ø) 1,000–2,000 nm (<1,000 nm) at frequency (dispersed particles) ranging of 0–55% and 0–63%, as showed in figures 11 (a) and (b). In analysis of particles distribution according to DLS method, in which particle size becoming more and more small, particles index (PI) is small. In other words, dispersed particles are more uniform and homogen. Both types of biomasses have particles index (PI) larger than 1 showing not uniform particles size [10], so that a emulsification and homogenization processes are needed to reduce particle size in order to ease adsorption of digestive system.
4. Conclusion

The utilize of folate-producing *L. acidophilus* and *B. brevis* L. species can be regarded as a new perspective on the specific uses of probiotics and are expected to produce folate in the presence of preformed pABA. Fermentation process of LAB using corn inoculum of *L. acidophilus* and *B. brevis* with inoculum specification and concentration, and different types of corn substrates generate biomass of fermented corn with different characteristic on composition, recovery of folic acid monomer, and distribution of particle size. Inoculum concentration becoming more and more increase will increase folic acid, total sugars, and total acids, however decreases total solids, reducing sugars, and dissolved protein for 4 (four) types of biomass. Based on optimization of folic acid, the best experiment treatment of fermentation on nixtamalized white corn and nixtamalized yellow corn were achieved by inoculum B and inoculum D with *B. brevis*, without and with enriched skimmed milk, and sucrose concentration 40 %. In these conditions, biomass B and biomass D have compositions of folic acid 103.07 and 91.92 µg/mL, dissolved protein 0.15 and 0.20 mg/mL, total sugars 70.27 and 92.95 mg/mL, reducing sugars 23.37 and 26.94 mg/mL, and total solids 7.53 and 20.97 %, respectively. In these conditions, it is occurred a increasing folic acid 508.4 % (5 times) and 433.18 % (4.33 times), and reducing sugars 86.07 and 116.38 % (1.16 times), however it is happened a declining dissolved protein 75.81 and 45.45 %, total sugars 18.29 and 5.15 %, and total solids 83.51 and 67.23 % compared with these components in nixtamalized white corn and nixtamalized yellow corn without fermentation. Biomasses B and D have particle size 987.4 nm and 762.8 nm with Index particles 2.384 and 2.085 dominated by folic acid monomer with MW 443.70 and 442.96 Da., and relative intensities 100 %, respectively.

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