Fermentation of Spinach (*Amaranthus* spp) and Broccoli (*Brassica oleracea* L.) Using Kombucha Culture as Natural Source of Folic Acid for Functional Food

Hakiki Melanie*, Agustine Susilowati, Yati Maryati and Puspa D. Lotulung

Research Center for Chemistry, Indonesian Institute of Sciences (LIPI)
Kawasan PUSPIPTEK Serpong, Tangerang Selatan 15314
** Corresponding author, tel/fax: 021-7560929/, email: hakiki.melanie@lipi.go.id*
Co-author 1, email: agustine_1408@yahoo.co.id
Co-author 2, email: maryati97@yahoo.com

ABSTRACT

The importance of folate (folic acid, B9 vitamin, pteroyl-L-glutamic acid) in human diet for the prevention of neural tube defects during pregnancy is widely known. Green vegetables are known to be a potential source of natural occurring folic acid instead of legumes and grains. The fermentation process using a mixed culture of kombucha is an alternative of natural folic acid production from green vegetables. Broccoli and spinach was selected based on its folic acid content compared to other vegetables. This research was conducted by the extraction of broccoli and spinach at 80 °C with a ratio of vegetable and water of 1:4. The inoculum was obtained by inoculating kombucha culture on vegetable extracts for 7 days at room temperature. The vegetable extracts were then fermented using inoculum with concentration of 15, 25 and 35% (v/w of polyphenol of vegetable extracts) for 0, 3, 6, 9, 12 and 15 days at room temperature. The analysis were performed on total solids, polyphenol, dissolved protein, reducing sugars, amylase and protease activities, total acid, total plate counts and folic acid contents. This study showed that the type of vegetables, inoculum concentration and fermentation time affect the chemical composition of fermented vegetables. Fermented broccoli and spinach with inoculum concentration of 15% had the highest yield of folic acid of 69,52 µg/mL and 62,05 µg/mL after 6 days and 3 days of fermentation time, respectively. Identification of folic acid in fermented vegetable extracts at optimum condition by LC-MS were carried out with the relative intensity for fermented spinach and broccoli were 0,48% and 0,56%, respectively, with a molecular weight of 459 m/z are identified as 5-methyl tetrahydrofolate.

Keywords: green vegetables; folic acid; kombucha culture; fermentation; LC-MS

ABSTRAK

Peranan folat (asam folat, vitamin B9, asam pteroyl-L-glutamat) dalam makanan untuk pencegahan neural tube defects (NTD) selama kehamilan telah banyak diketahui secara luas. Sayuran hijau merupakan sumber asam folat alami yang potensial selain dari kacang-kacangan dan biji-bijian. Proses fermentasi menggunakan kultur campuran kombucha merupakan alternatif untuk perolehan asam folat pada sayuran hijau. Pemilihan atas brokoli dan bayam berdasarkan komposisi terutama asam folat yang lebih tinggi dibandingkan dengan jenis sayuran lainnya. Penelitian dilakukan dengan ekstraksi brokoli (Brassica oleracea L.) dan bayam (Amaranthus spp.) pada suhu 80ºC dengan rasio sayuran dan air 1:4. Inokulum sayuran diperoleh dengan menginokulasi kultur kombucha pada ekstrak sayuran selama 7 hari pada suhu ruang. Perolehan bubur dari hasil ekstraksi sayuran selanjutnya difermentasi menggunakan inokulum sayuran dengan konsentrasi 15, 25 dan

35% (v/b polifenol ekstrak sayuran) selama 0, 3, 6, 9, 12 dan 15 hari pada kondisi tetap (suhu ruang dan konsentrasi sukrosa 10%). Analisis dilakukan terhadap total padatan, polifenol, protein terlarut, gula reduksi, aktivitas amilolitik dan proteolitik, total asam, TPC dan asam folat. Hasil penelitian menunjukkan bahwa jenis sayur, konsentrasi inokulum dan waktu fermentasi berpengaruh terhadap komposisi sayuran terfermentasi. Sayuran terfermentasi dengan konsentrasi inokulum 15% memberikan perolehan asam folat terbaik yaitu sebesar 69,52 ug/mL pada brokoli setelah 6 hari terfermentasi dan 62,05 ug/mL pada bayam setelah fermentasi selama 3 hari pada suhu ruang. Identifikasi asam folat pada ektrak sayuran bayam dan brokoli pada kondisi optimum menunjukkan intensitas relatif berturut-turut sebesar 0,48% dan 0,56% masing-masing dengan berat molekul 459 M/Z yang diidentifikasi sebagai 5-methyl tetrahydrofolat.

Kata Kunci: sayuran hijau; asam folat; kultur kombucha; fermentasi; LC-MS

I. INTRODUCTION

Folate presents as an essential group among the vitamin B family, which is taking part in one carbon transfer reaction required within the cell. Folates are tripartite molecules consisting of pteridine, p-aminobenzoate (PABA) and glutamate moieties usually with a short, γ-linked chain of additional glutamates attached to the first glutamate [1]. The main compound of this group is based on pteroic acid [2]. Folic acid is a common form of folates, which is including in the vitamin B family. The native folates consist of two or four additional hydrogens in their pteridine ring forming dihydro- or tetrahydrofolates. Meanwhile, dietary folates present primarily as reduced forms of one-carbon substituted of pteroyl [3], [4]. Folates have an essential function in human especially for purine and pyrimidine biosynthesis (DNA and RNA), synthesis of the genetic materials of cells, as well as for protein metabolism [5].

Folate deficiency in diets has been associated with the malfunction of the embryonic brain development, referred as neural tube defects (NTDs). Furthermore, it also linked to anemia, artherosclerosis, Alzheimer disease, stroke and cancers [6]. Folate cannot be synthesized by humans or mammals and must be obtained through diet. Therefore, naturally folate intake through diet should be increase by consumption of folate rich foods. Folate can be found in green leafy vegetables, fruits, egg yolk and legumes but are insufficient source in most of the cereals and their flours [7]. The health benefits of incorporating a wide variety of vegetables in the diet are well-recognised. Green leafy vegetables are reported to be excellent sources of folates which are water-soluble vitamins and occur naturally in foods in many forms or vitamers. It is important to provide consumers with as much information as possible on natural sources of folate, which are more effective than supplementation or fortification with folic acid [8].

Fermentation in vegetables using culture kombucha is believed to be capable of generating folic acid content, as well as in fermented tea leaves (green) which increases the content of L-theanine (Ethyl-L-glutamine or L-glutamic acid γ- ethylamide) and total polyphenols [9]. Presumably this change is caused by the activity of the enzyme invertase of microbes (bacteria, yeasts, molds) on the kombucha culture which is symbiotic partnership of yeast and bacteria such as *Acetobacter xylinum*, *Saccharomyces cereviseae, Saccharomyces ludwigii, Saccharomyces bisporus, Zygosaccharomyces* sp and *Torolupsis* sp [10]. It was also proved that the process of fermentation improves the synthesis of B vitamins and folic acid [11].

The purpose of the present study was to determine the optimized fermentation process of green vegetables (spinach and broccoli) with various inoculum concentrations using Kombucha culture for the concurrent production of natural folic acid to be used in functional food formulation.

II. EXPERIMENTAL SECTION

2.1 Materials

Materials used in this study were green vegetables (spinach and broccoli) obtained from local market and kombucha culture. All chemicals and solvents used for process and analysis were of analytical grade and purchased from local distributors.

2.2 Instrumentation

Process apparatus used in this experiment were microbiology equipment such as laminar air flow, water bath, incubator, fermenter, high separation frequency, homogenizer, grinder, vacuum drier and cabinet. The main analysis instruments were spectrophotometer UV-Vis and LC-MS.

2.3 Experimental Design

This experiment was conducted by fermenting green vegetables with condition variation as follow, (1) types of green vegetables (broccoli and spinach); (2) kombucha culture concentration; (3) fermentation time from 0 to 15 days with intervals of 3 days for sampling. Analysis was done for total solids (gravimetry), dissolved protein (Lowry), N-amino (Cu method), protease activity (Kunitz), amylase and reducing sugar (Somogy-Nelson) and folic acid (spectrophotometry). Furthermore, folic acid oligomer was identified by LC-MS (Mariner Biospectometry) with LC (Hitachi L6200). All samples were conducted in duplicate and all data were processed in descriptive analysis based on average result of analysis.

2.4 Procedures

Extraction of vegetables through blanching process

A number of green vegetables (spinach and broccoli) were blanched for 15 minutes at 80ºC. The vegetables from blanching process were crushed in various ratio of vegetable and water (1:1, 1:2, 1:3 and 1:4) and green vegetables suspension were then obtained.

Preparation of vegetable-kombucha inoculum

The suspension of green vegetables from blanching process was filtered through 80 mesh sieve. The filtrate of green vegetables was then added with sucrose (10% b/v of vegetable filtrate), folic acid and kombucha culture (10%) and fermented in enclosed container for 7 days in dark room. The inoculum was obtained after 7 days of fermentation.

Fermentation process of green vegetable with vegetable-kombucha inoculum

The filtrates of green vegetables from blanching process were used as substrate in the fermentation process. Filtrates were added with inoculum of 15%, 25% and 35% (v/v of vegetable filtrate), respectively and sucrose 10% (b/v of vegetable filtrate) and stored in container with aeration (covered with cotton cloth) in dark room at room temperature for 0, 3, 6, 9, 12 and 15 days. The entire work was done aseptically. The accumulation of biomass after fermentation was obtained as fermented vegetable suspension.

Analysis of folic acid

Folic acid analysis was performed using spectrophotometric method based on diazotization reaction of p-aminobenzoylglutamat acid produced after reduction reaction of folic acid and 3-aminophenol to form an orange-yellow complex. The standard folic acid or sample solution of 1,0 mL were mixed with to 1,0 mL of 4 mol L-1 HCl, 1,0 mL of 1% (w/v) sodium nitrite, 1,0 mL of 1% (w / v) sulfamic acid and 1,0 mL of 1% (w / v) 3-aminophenol which was resulting in an orange-yellow complex. The absorption of complexation was measured at 460 nm using UV-Visible spectrophotometer [12].

Identification of folic acid by LC-MS

After fermentation process, fermented vegetables were then filtered by microfiltration. The obtained permeate or purified fermented vegetables extracts and folic acid standard were then analyzed by LC-MS. The analysis of folic acid oligomer was conducted by LC-MS using Mariner Biospectrometry. The spectrometer was operated in scan mode (100-1200m/z) using positive electro spray ionization (ESI). Chromatographic separation was carried out on Supelco C18 analytical column (250mm x 2 mm, 5 µm particle size). Mobile phase consisted of aqueous acetic acid (0,3%) and methanol containing acetic acid 0,3% at a flow rate of 1,0 mL/min. An injection volume of 20 µL was used in this analysis [13].

III. RESULTS AND DISCUSSION

3.1 Chemical Characterization of Green Vegetables

The selection of spinach and broccoli as natural folic acid source in food formulation due to its source is easily obtained and consumed in food processing. Based on proximate analysis of spinach and broccoli in Table 1, it is known that spinach and broccoli are potential as a source of natural folic acid to be utilized in the next fermentation process. This is due to high chlorophyll content in those vegetables. Chlorophyll is protein source and potential for folic acid source. Chlorophyll function in photosynthesis triggers the fixation of carbon dioxide to produce carbohydrate and provide energy for the entire ecosystem. Carbohydrate produced in photosynthesis is converted into proteins, lipids, nucleic acids and other organic molecules. This will be the basis that the high chlorophyll concentration will yield folic acid at optimum concentration through fermentation of green vegetables inoculated with Kombucha culture.

Figure 1 and 2 show the fermented spinach and broccoli extract from the start and the last day of fermentation using vegetable inoculum with kombucha.

Table 1 Chemical composition of spinach and broccoli

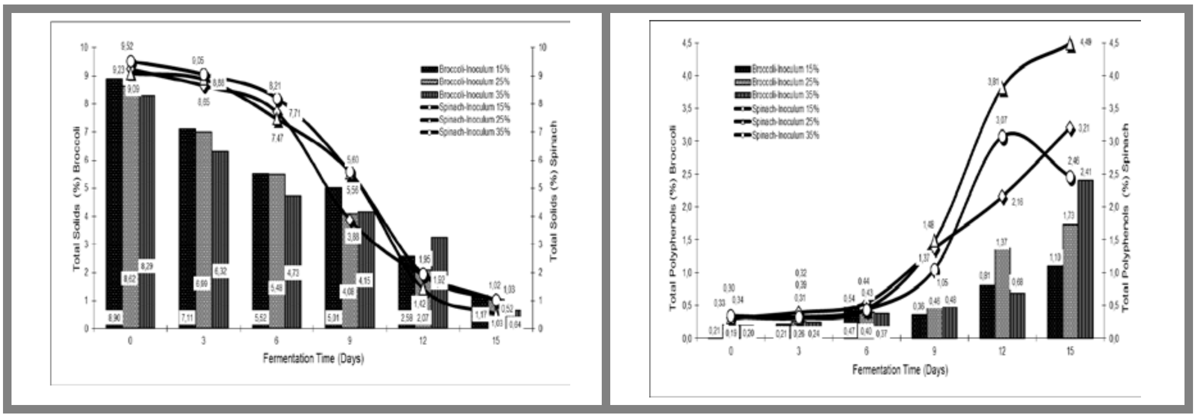
Composition	Spinach	Broccoli
Moisture Content (%)	18,34	25,51
Total polyphenol (%)	0,20	0,02
Chlorophyll (mg/L)	10,26	3,90

(a) (b) (c) (d) (e) (f)

Figure 1 Fermented spinach extract with 15% Kombucha-vegetable inoculum in 0 (a), 3 (b), 6 (c), 9 (d), 12 (e) dan 15 (f) days of fermentation time at room temperature

(a) (b) (c) (d) (e) (f)

Figure 2 Fermented broccoli extract with 15% Kombucha-vegetable inoculum in 0 (a), 3 (b), 6 (c), 9 (d), 12 (e) dan 15 (f) days of fermentation time at room temperature



(a) (b)

Figure 3 Effects of fermentation time and inoculum concentration on total solids (a) and total polyphenols (b) of fermented spinach and broccoli using Kombucha culture

3.2. Effect of Fermentation Time and Inoculum Concentration on Composition of Fermented Vegetables using Kombucha Culture

Total solids and total polyphenol

Fermentation of vegetables extract using kombucha inoculum at room temperature gave higher total solids of fermented spinach extract than broccoli extract. Fermentation time affects the recovery of total solids, as the longer fermentation time the lower total solids content were obtained for both fermented spinach and broccoli extract. Total solids content of fermented spinach and broccoli extract decrease from 9,23% to 1,03% and 8,90% to 1,17% after 15 days of fermentation, respectively. The reduction in total solids content during fermentation was caused by the increasing activity of microbes during fermentation process since microorganisms require more water and nutrients from bioactive compounds for their metabolism.

Changes in total polyphenol obtained during fermentation are shown in Figure 3b. Total polyphenol contents were increased linearly with fermentation time and fermented spinach and broccoli had higher polyphenol content at day 15. The amount was accumulated up to 4,49% and 2,41% at day 15 of fermentation time for spinach and broccoli respectively. Polyphenol compounds are called high-level antioxidants because of their ability to scavenge free-radical and active oxygen species. Complex polyphenol compound in fermented vegetables might be subjected to degradation in acidic environment of kombucha and by the enzymes liberated by bacteria and yeast in kombucha fungus consortium. So, there are many chances for the enzymes liberated by bacteria and yeast during kombucha fermentation will be the reason for the degradation of complex polyphenols to small molecules which in turn results in the increase of total phenolic compounds [14]. In addition, since the colour of fermented vegetables broth with kombucha continued to get lighter, this suggested that polyphenols did undergo microbial transformation because of the suppression of ionization or destruction of structures [15]. It was therefore possible that kombucha starters secreted some unknown enzymes that were capable of catalyzing the biodegradation of polyphenols. However, quantity of total polyphenol content did not always determine the antioxidative activities whereas the types of metabolites produced might have the key effect [16].

Amylase activity and reducing sugar

Yeasts and bacteria in kombucha are involved in such metabolic activities that utilize substrates by different and in complementary ways. Sucrose is the most common carbon source in kombucha fermentation. Yeasts hydrolyze sucrose into glucose and fructose by invertase and produce ethanol via glycolysis, with a preference for fructose as a substrate [17].

Amylase activity in fermented vegetables using kombucha culture indicates the ability of microbes to hyrolyze carbohydrate chain into smaller disaccharides units. Figure 4a describes the amylase activity of fermented vegetables is quite fluctuate, however the optimum condition of fermented spinach and broccoli reached on sixth and third day of fermentation time, which were 2581,20 U/mL and 2629,17 U/mL, respectively. Previous reports showed that amylase could be inhibited by monomeric phenolic compounds. Thus, the decrease in amylase activity could be due to the phenolic fraction produced in fermented vegetables using kombucha culture [18]

Amylase activity of microbes is closely related to the reduction of sugar produced during fermentation. Sucrose is hydrolyzed by Kombucha invertases into glucose and fructose, which are used as a carbon source of the symbiotic system. Reducing sugar is a parameter of monosaccharide formation hydrolyzes by amylase enzyme. Based on Figure 5b, reducing sugar during fermentation is fluctuating. However, reducing sugar reached optimum at third day and sixth day of fermentation for fermented broccoli and spinach, which are 296.63 mg/mL and 291.19 mg/mL, respectively. This is linear with optimum condition of amylase activity obtained during fermentation of green vegetables.

Protease activity and dissolved protein

As the extent of proteolysis is mainly depends on the protease enzymes of starter cultures [19]. Protease activity during fermentation of vegetables showed the ability of protease enzymes to break down proteins into smaller amino acid units. In fermented spinach, optimum protease activity is obtained at the early phase of fermentation (0,99 units/mL) and then continued to decline until the 15th day of fermentation. Meanwhile fermented broccoli obtained optimum protease activity (0.73 U/mL) on the 6th day of fermentation and further decreased to 15th day of fermentation. Differences in optimum condition of protease activity during fermentation are possible due to influence of several environmental factors such as moisture, oxygen distribution, fermentation temperature and stirring homogeneity. Based on physical appearance, the color of fermented spinach extract was darker than broccoli thus allowing inhibition of oxygen distribution and the increasing temperature, as microorganisms in kombucha culture work well in anaerobic condition, therefore protease enzyme was immediately activated at the beginning of fermentation. However, lower protease activity in fermented vegetables may be considered as the result of symbiosis occurring among the microbe populations. Thus, instead of being accumulated, they are further consumed [20].

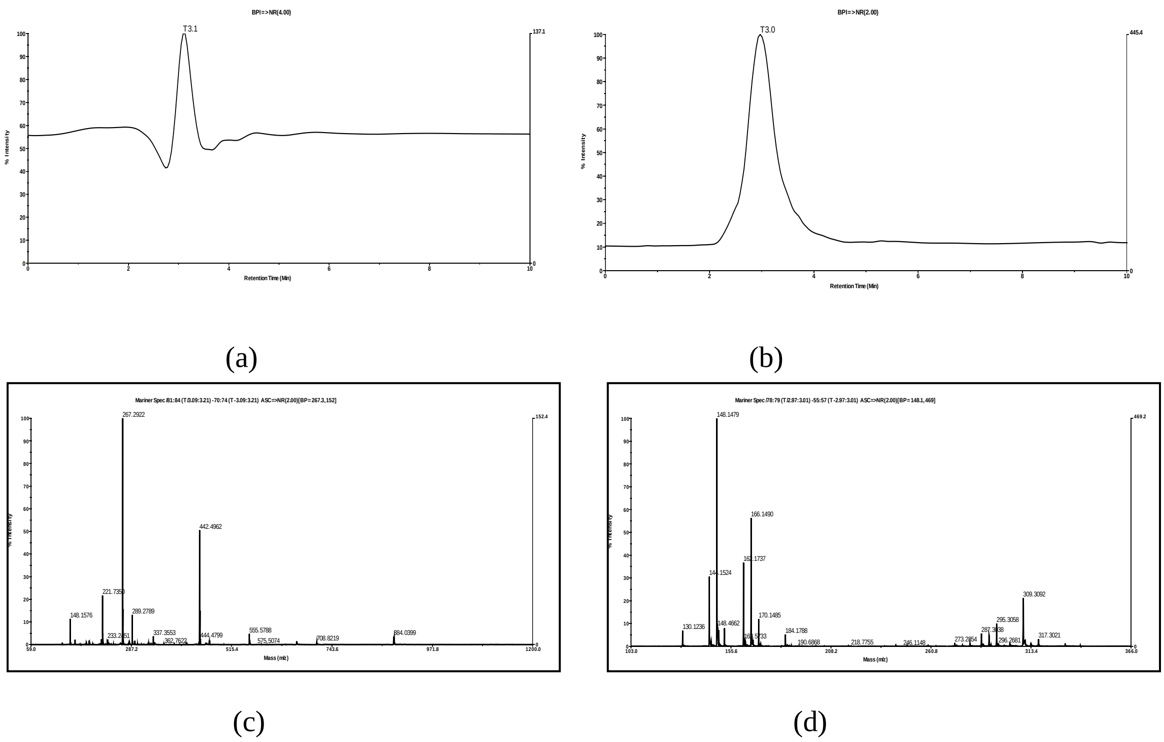
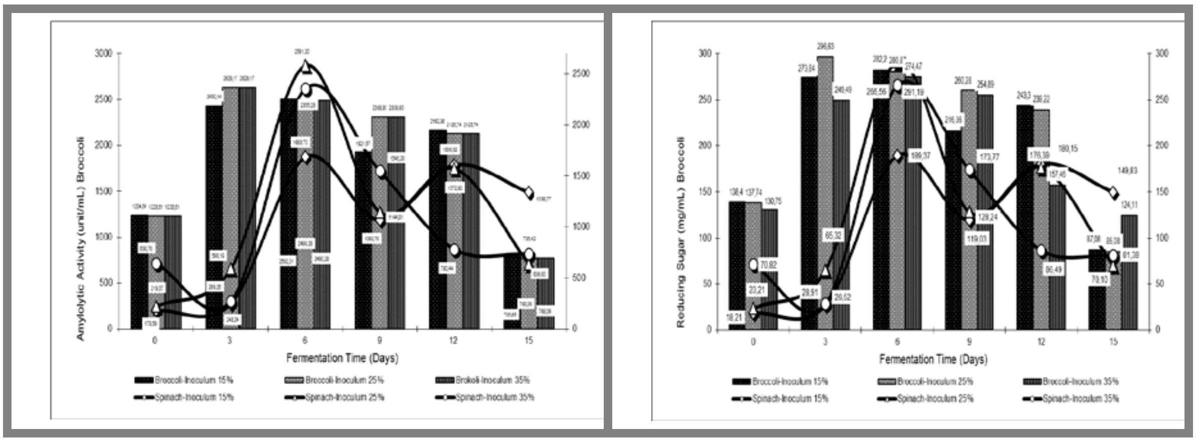


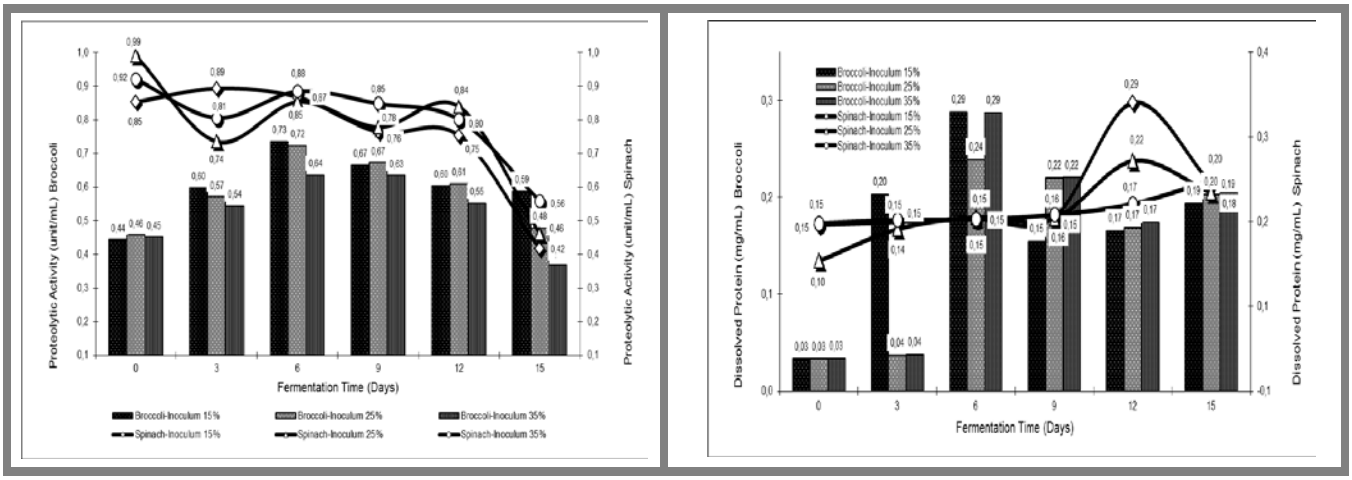
Figure 4 LC-MS analysis of folic acid (a) and glutamic acid (b) standard. The MS spectra of folic acid (c), and glutamic acid (d) standards.



(a) (b)

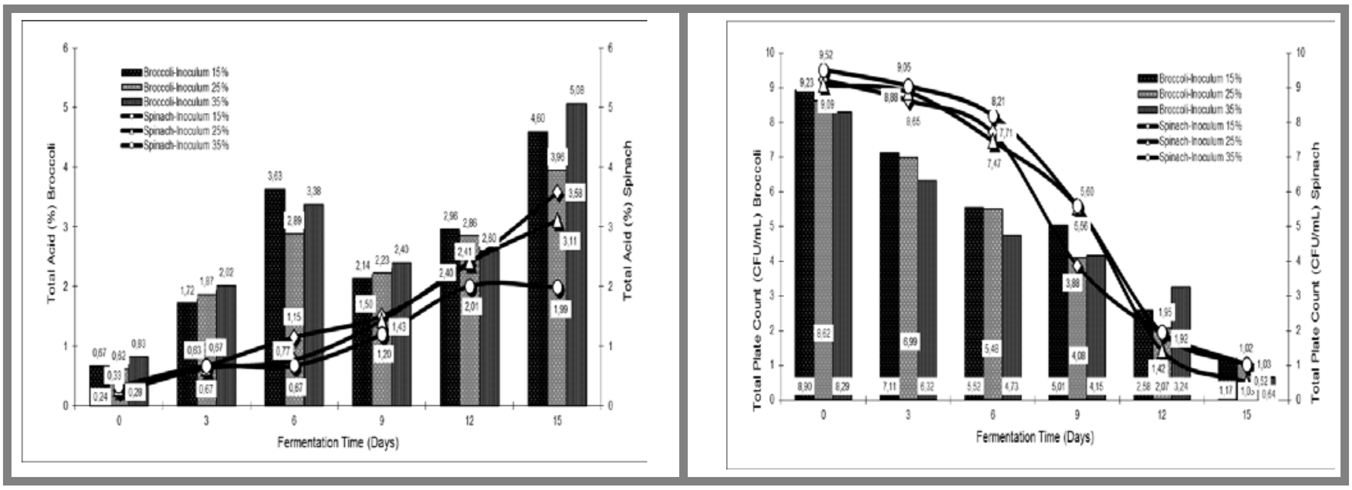
Figure 5 Effects of fermentation time and inoculum concentration on amylolytic activity (a) and reducing sugar (b) of fermented spinach and broccoli using Kombucha culture

Dissolved protein in vegetables fermentation is a parameter of protein breakdown with large molecular weight into amino acids and peptides with lower molecular weight by protease resulting in dissolved protein with high solubility. Figure 6b indicates that dissolved protein content increased with fermentation time. These increasing proteins likely represent extracellular protein secreted by bacteria and yeasts during the fermentation time [21]. Afterwards, it continued to decrease because of yeast and bacterial extracellular protein decreases [17]. Dissolved protein reached optimum condition at 0,29 mg/mL for both fermented broccoli and spinach on the 3rd and 12th day of fermentation time, respectively.



(a) (b)

Figure 6 Effects of fermentation time and inoculum concentration on proteolytic activity (a) and dissolved protein (b) of fermented spinach and broccoli using Kombucha culture



(a) (b)

Figure 7 Effects of fermentation time and inoculum concentration on total acids (a) dan total plate count (b) of fermented spinach and broccoli using Kombucha culture.

Total acids and total plate count

Acetic acid bacteria are the most popular microorganism found in Kombucha culture. Acetic acid stimulates production of ethanol which, in turn, may facilitate the growth of acetic acid bacteria and production of acetic acid [22]. Acetic acid bacteria initially oxidises ethanol to acetic aldehyde and then to acetic acid. This bacteria alsoactivate glucose oxidation to gluconic acid [11]. Total acids is the essential parameter in fermentation using Kombucha culture, whereas Kombucha culture will produce several acid compounds such as acetic acid, lactate acid, gluconate acid and glucuronate acid. By the increasing of fermentation time, acid production will increase as shown in Figure 1a. Total acids yield in 5,08% and 3,58% for fermented broccoli and spinach on the 15th day of fermentation.

Total acid obtained during fermentation affects microbe total counts and polyphenol content. Based on Figure 7b, the viable counts of yeasts decreased gradually in the latter period of fermentation. Total microbial counts obtained at the end of fermentation for broccoli and spinach are 0.64 and 0.52 CFU/mL, respectively. The decrease in microbial population at the end of fermentation is caused by a reduction in carbon and antimicrobial compounds found in green vegetables including organic acid compounds resulting from polyphenols. Furthermore, the carbon dioxide generated as a result of alcohol fermentation by yeasts accumulated in the interface between the pellicle and broth; this separated the pellicle from the broth and eventually, blocked the transfer of nutrients from the broth to the top and the transfer of oxygen from the surface of the pellicle. These two deleterious effects led to an anaerobic and starved environment. Few genera of yeasts and bacteria could survive such conditions. Therefore, viability of both yeasts and aerobic acetic acid bacteria decreased gradually during fermentation [23].

Folic acid

Fermentation in vegetables using Kombucha culture is potentially considered to generate natural folic acid as in fermentation of tea which increases L-theanine and polyphenol content [9]. Presumably, this is caused by the activity of invertase enzyme from microbes (bacteria, yeast, mold) in Kombucha culture, which is a symbiotic collaboration of yeast and bacteria such as *Acetobacter xylinum*, *Saccharomyces cereviseae*, *Saccharomyces ludwigii*, *Saccharomyces bisporus*, *Zygosaccharomyces* sp and *Torolupsis* sp (Malbasa, et al., 2008). It was also proved that the process of fermentation improves the synthesis of B vitamins and folic acid [11].

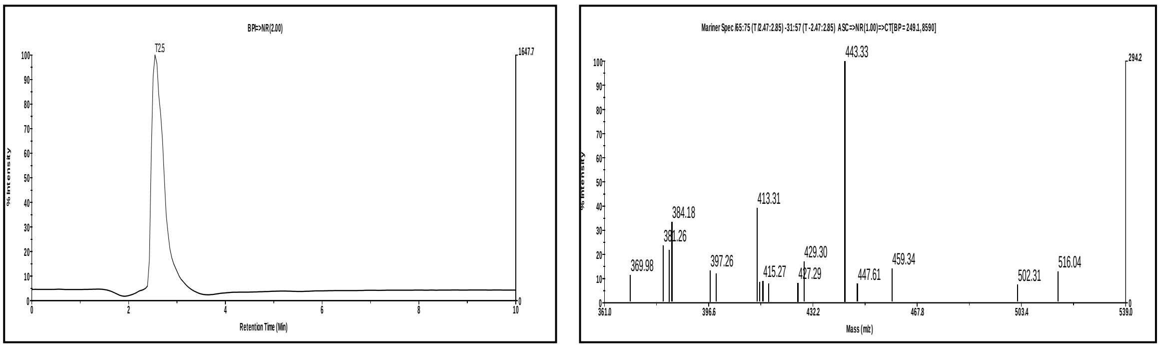
Folic acid at optimum condition was obtained on the 3rd and 6th day of fermentation at 15% of inoculum concentration for spinach and broccoli, which were 62,05 and 69,52 µg/mL respectively. Folic acid is a highly sensitive compound to light, oxygen and temperature, therefore fermentation using kombucha culture could increase recovery of natural folic acid since it is conducted in dark room and anaerobic conditions to minimize the interaction with light and oxygen. In addition, microbial activitiy in kombucha culture is expected to help the stability of natural folic acid during fermentation.

Identification of folic acid by LC-MS

Identification of folic acid in fermented spinach and broccoli extract were performed by LCMS on optimum condition based on folic acid content. Identification is conducted by using standard of folic acid and glutamic acid as shown in Figure 4a and 4b. Figure 4c shows that folic acid standard is dominated by compounds with molecular weight of 442.5, 443.16, 443.51 and 444.48 m/z respectively. In Figure 4d, glutamic acid standard is dominated by compounds with molecular weight of 148.15, 148.47 and 149.14 m/z respectively.

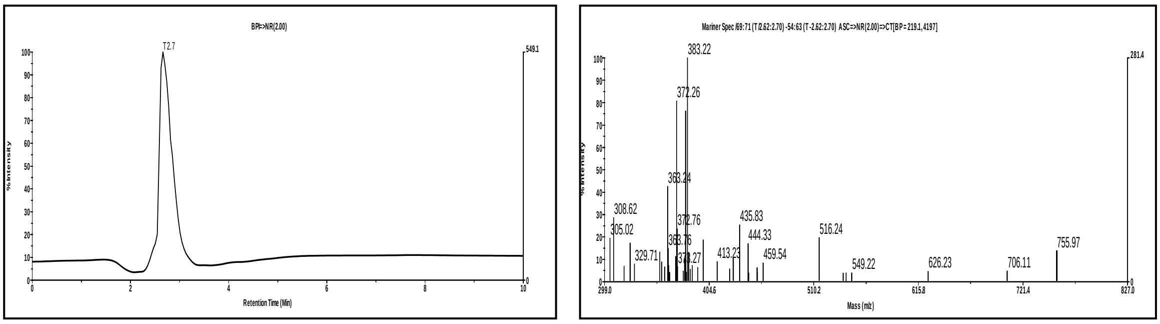
Identification of folic acid in fermented spinach and broccoli extract prepared by diluting 1 part of fermented vegetable extract with 4 parts of water and then filtered through microfiltration stirred cell at pressure of 40 psia and stirrer rotational speed of 400 rpm. The results show that the identification of folic acid by LC-MS as T 2.5 in Figure 8a, while Figure 8b respectively show the mass spectra of T 2.5 chromatogram.

Figure 8b showed the positive ion electrospray mass spectrum of folic acid in fermented spinach extract at molecular weight of 427, 443, 447 and 459 m/z represented molecules of 2-deaminofolic acid, dihydrofolate, 5,6,7,8-tetrahydrofolate and 5-methyl tetrahydrofolate with intensities of 0,28; 3,42; 0,27 and 0,48% respectively. Cellular folate species are differentiated by the reduction state of the pteridine ring (tetra- or dihydrofolate), identity of the one-carbon substituent at the N 5 and/or N



(a) (b)

Figure 8 LC-MS analysis of folic acid in fermented spinach extract with retention time 2,5 minute (a), MS spectra illustrating the sequential detection of folic acid (b)



(a) (b)

Figure 9 LC-MS analysis of folic acid in fermented broccoli extract with retention time 2,7 minute (a), MS spectra illustrating the sequential detection of folic acid (b)

10 positions (formyl, methyl, methylene, methenyl and formimino), and the length of the γ-glutamyl chain. It was found that the 5-methyl tetrahydrofolate is the major folate form in plants and accounts for 28–90% of total folate [1]. Formyl derivatives account for 30–55%, and tetrahydrofolate and methylene derivatives 10–15%. At the subcellular level, folate is distributed in different plant cell compartments (mainly cytosol, vacuole, mitochondria and chloroplasts) in polyglutamyl forms [24], [25].

The low folic acid intensity caused by the degradation of folic acid during extraction and fermentation process as folic acid is known to be very sensitive to temperature, light and oxygen. The peak at 190, 207, 263 and 312 m/z represented the degraded products of folic acid due to photolysis reaction, which are 6-formylpterin, pterin-6-carboxylic acid, p-amino-benzoyl-L-glutamic acid and pteroic acid [26].

Folic acid analysis by LC-MS in fermented broccoli extract is shown at Figure 9a at retention time of 2,7 minute. Figure 8b showed the positive ion electrospray mass spectrum of folic in fermented broccoli extract at molecular weight of 426, 444, 453 and 459 m/z represented molecules of 2-deaminofolic acid, 5,6,7,8-tetrahydrofolate, 5,10-methenyltetrahydrofolate and 5-methyl tetrahydrofolate with intensities of 0,38; 1,14; 0,42 and 0,56% respectively. The degraded product of folic acid due to photolysis were also observed in this mass spectra at peak of 192, 207 and 263 m/z represented 6-formylpterin, pterin-6-carboxylic acid and p-amino-benzoyl-L-glutamic acid [26].

IV. CONCLUSION

Type of vegetables, fermentation time and inoculum concentration affect the composition of fermented vegetables using Kombucha culture. Fermented vegetables extract with inoculum concentration 15% attained optimum folic acid content of 69,52 and 62,05 µg/mL in broccoli and spinach after 6th day and 3rd day of fermentation at room temperature, respectively. Liquid chromatography directly combined with electrospray-ionization mass spectrometry can be used to selectively separate and detect folic acid and its degradation products due to photolysis in fermented vegetables. Both spinach and broccoli represented 5-methyl tetrahydrofolate, which is a major form of folic acid found in vegetables.

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