Characteristic of Fermented Spinach (*Amaranthus spp.*) Polyphenol by Kombucha Culture for Antioxidant Compound

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Abstract. Fermentation on spinach (*Amaranthus* sp.) vegetable by kombucha culture as an effort to get poliphenol as antioxidant compound had been done. Purification of fermented spinach extract suspension was carried out through microfiltration (MF) membrane (pore size 0.15 μm) fitted in dead-end Stirred Ultrafiltration Cell (SUFC) mode at fixed condition (stirrer rotation 400 rpm, room temperature, pressure 40 psia). Result of the experimental activity showed that long fermentation time increased total acids, total polyphenol and Total Plate Count (TPC), and decreased total solids and reducing sugar in biomass. The optimal fermentation time was reached for 2 weeks with total polyphenol recovery increasing of 92.76 % from before and after fermentation. On this optimal fermentation time, biomass had identified galic acid with relative intensity of 8 %, while as polyphenol monomer was resulted 5 kinds of polyphenol compounds with total intensity 27.97 % and molecular weight (MW) 191.1736, 193.1871 and 194.2170 at T2.5, T2.86 and T3.86. Long fermentation time increased functional properties of polyphenol as antioxidant.

INTRODUCTION

Vegetables fermented by kombucha culture is a novelty fermentation process as a main effort in order to get bioactive components based on nutrition need for anti oxidant. Fermentation on vegetables had been recognized and consumed sufficient long**,** such as kimchi, kombucha tea, pickle, salted vegetables (fermented collard, fermented cabbage), which is performed with initial aim in order to preserve foods, and get both specific vegetable taste and aroma. Potency of spinach (*Amaranthus* sp.) as an anti-oxidant has recently been explored.

Spinach as phytochemical on phenolic compounds (phenolic acids and flavonoid) has anti-oxidant properties. Via fermentation process using kombucha culture, phenolic compounds expressed as total polyphenol has possibility higher concentration due to invertase enzyme activity from kombucha culture. Process condition of fermentation (both temperature and time) affects on total polyphenol. Figure 1 shows chemistry structure of Epigallocatechin-3-gallate (EGCG).

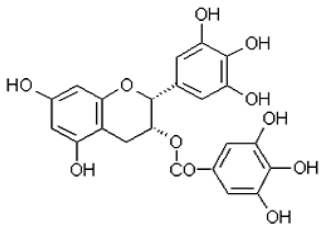

FIGURE 1. Chemistry structure of Epigallocatechin-3-gallate

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Kombucha culture contains acetate acid bacteria, such as *Acetobacter xylinum* and yeast (*Saccharomyces cereviceae*, *Saccharomyces ludwigii*, *Saccharomyces bisporus*, *Zygosaccharomyces* sp.) and several kinds of yeast (*Torolupsis* sp.) [1]. A symbiosis of bacteria and various fungi will produce lactic acid, vitamins, amino acids, antibiotic and other components ([2]. Spinach fermentation by kombucha culture with long fermentation time enables to be get spinach polyphenol with low molecular weight (MW) because of invertase enzyme activity of kombucha culture. Purification of spinach polyphenol is conducted by means of microfiltration (MF) membrane (pore size 0.15 μm) based on particles size relating with MW. MF membrane allows freely the passage of polyphenol compounds as permeate (extract) because they have MW ranging from 200 to 600 Dalton (Da.) or particles size ranging from 0.001 to 0.1 μm) [3], such as organic acids, amino acids, vitamin and mineral [4]. MF is able to separate macromolecules with MW larger than 500.000 Da. or particles size range larger than 0.1 – 10 μm [5].

To know characteristic of polyphenol compounds in biomass is carried out identification by *Liquid Chromatography coupled with Mass Spectrometry* (LC-MS), which enables to be known range of polyphenol MW due to its functional properties. LC-MS is a hybrid of *chromatography* and *mass spectrometry*. By using *chromatography*, it will separated molecular mixture based on difference in migration speed and molecules distribution in (adsorbent) and eluent, while *mass spectrometry* will ionize analyte based on prinsiple of *electro spray ionisation* (ESI) to gas phase (fine aerosol) [6]. LC-MS will separate polyphenol monomer and identify MW. Polyphenol MW becomes more and more low, its functional properties as anti-oxidant becomes more and more high.

The objective of this experimental work was find out compounds characteristic yielded via spinach fermentation by kombucha culture under difference in fermentation time purified by means of dead-end Stirred Microfiltration Cell (DESMFC) mode on composition and molecular weight (MW) using LC-MS instrument.

MATERIALS AND METHODS

Materials and Equipments

Main materials used in this experimental activity were fresh green spinach (*Amaranthus* spp.) leaves obtained from local market, kombucha culture (Pusat Penelitian Kimia - LIPI), commercial fluoro polymer microfiltration (MF) membrane (pore size 0.15 μm, FSM-0.15-PP, Danish Separation Systems, Denmark), distilled water, sucrose, and chemicals for preparation and analysis purposes. All of the chemicals with analytical grade quality.

Main equipments utilized in this experimental work were high precision balance (Fujitsu, Japan), shaker (Memmert, Germany), distilling unit (Gesellschaft fur Labortechnik GmbH/GFL, Type 2012, Germany), thermometer, porcelain mortar & pestle (Z247529-1EA, SIGMA-ALDRICH), homogenizer (Ultra-Turrax, Ika Labortechnik, T50, Jane & Kunkel, Germany), sieve of 80 mesh (Retsch, Germany), autoclave (CHENG YI, LS – 50 L, China), incubator (Local), system of laminar flow chamber (Local), series of fermentation system in laboratory scale (local), stop watch (Hanhart Profil 2, Germany), magnetic stirrer (HI 303 N, HANNA Instrument, Japan), pressure gauge of technical nitrogen (Fisher Scientific Company, England), cylindrical tank for technical nitrogen (Local), Dead-End Stirred Ultrafiltration Cell (SUFC) (MILLIPORE, Model 8200, U. S. A.), UV-Vis Spectrophotometer (Model RF-550, Shimadzu, Japan), LC-MS (Mariner Biospectrometry) with LC (Hitachi L 6200) [7] and glassware (erlenmeyer flask, beaker glass, cylinder glass, stoppered conical flask).

Experimental Design

Experimental work was carried out at fixed process condition (room temperature, kombucha culture concentration of 25 %, sucrose concentration of 10 %). Biomass of fermented spinach was then purified through MF membrane (pore size 0.15 μm) fitted in Dead-End Stirred Ultrafiltration Cell (SUFC) under fixed process condition (stirrer rotation 400 rpm, room temperature and pressure 40 psia for 30 minutes). Analysis were employed on feed, and permeate and retentate (as a result of purification) covering Total Solids (Gravimetric method), Total Sugars (Somogyi-Nelson method), Total Acids (Titration method) [8] and Total Polyphenol (Folin-Denis Method) [3].

Process Steps

Fermentation Process of Spinach (*Amaranthus* sp.)

Fresh green spinach leaves was washed in running water, blanched at 80 °C and 15 minutes, pulverized by adding water at a 1 part : 4 parts ratio of spinach to water, and filtered through 80 mesh sieve to result filtrate as substrate used further and residue. Filtrate was then autoclaved at 121 °C for 15 minutes, cooled and added kombucha culture of 15 % (v/v spinach filtrate) and sucrose of 10 % (w/v spinach filtrate). Sterilized filtrate was fermented in closed container followed aeration (cassa cloth) in dark room at room temperature for 15 days. The whole activity was carried out aseptically. Biomass obtained is fermented spinach extract suspension.

Purification of fermented spinach by kombucha culture through SMFC

Separation and purification of fermented spinach extract suspension were conducted by means of MF membrane (0.15 µm) fitted in dead-end SUFC mode. Process schematic of preparation and purification was shown in Figure 2.

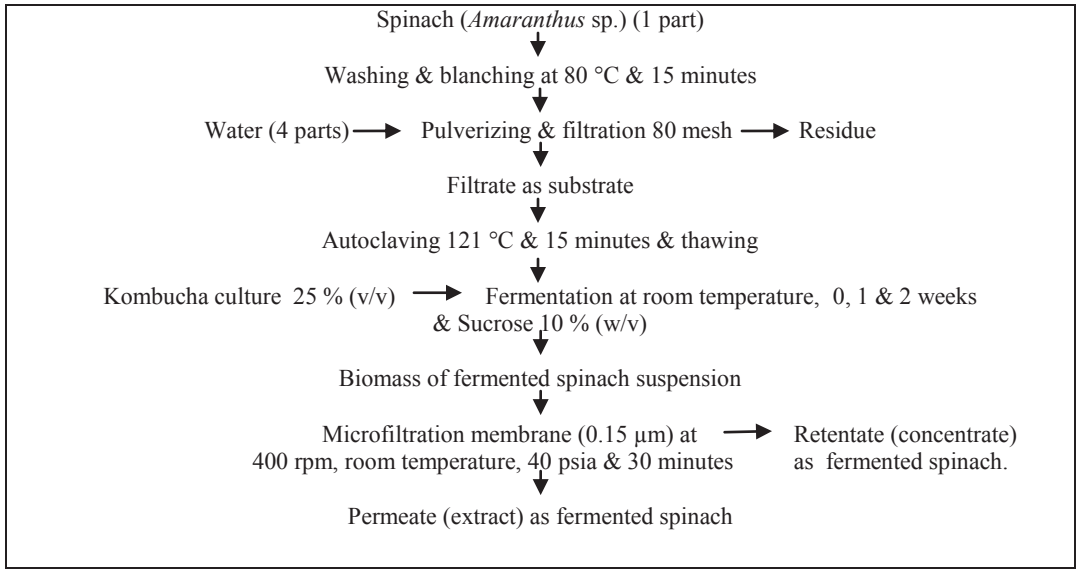

FIGURE 2. Scheme of separation process of polyphenol in fermented spinach through MF membrane (0.15 um) fitted in dead-end SUFC mode.

Before used, the cell containing a magnetic stirring bar and membrane was filled by 50 mL of distilled water to wet the membrane. Distilled water in the cell was then replaced with fermented spinach and operated at stirrer rotation speed of 400 rpm and pressure of 40 psia for ± 30 minutes by providing nitrogen gas from cylinder until a more and less permeate steady flux had been achieved. Permeate passing from membrane was collected and recorded its volume to calculate permeate flux value, and retentate or concentrate in the cell was discharded. Both permeate and retentate or concentrate were then analysed. After processed the membrane was washed with distilled water [9].

Identification of polyphenol compound by LC-MS instrument

Samples of permeate (extract) as a result of separation and/or purification on fermented spinach were identified via LC-MS using Mariner Biospectrometry. LC system was integrated with Q-tof mass spectrometer through Electro Spray Ionization (ESI) system, in which scan mode ranged from 100 to 1200 mz and 140 °C. LC (Hitachi L 6200) completed by C18 (RP 18) Supelco column with 250 mm of column length x 2 mm and particle size of 5 µm. Kind of solvent is water containing 0.3 % acetic acid (A) and methanol containing 0.3 % acetic acid (B) at a 90 parts : 10 parts ratio of methanol to water with flow rate of 1 mL/minute and injection volume of 20 µL [7].

Analysis of Total Polyphenol

Accurately, weight of biomass (2.0 g) is added to 200 mL boilling water into beaker glass, heated for 45 minutes in boilling water bath, and shaked the beaker glass every 10 minutes. At first filtered through gauze and allow the sample solution return to room temperature prior to dilute it to 250 mL with nanopure water. For preparationn of standard solution**,** 25 mg gallic acid into 250 mL volumetric volume, add nanopure water to volume and shake to mix. For determination, accurately pipette 1 mL prepared sample solution add 0, 1, 2, 3, 4, 5, 6,7, 8, 9 and 10 mL standard solution into 100 mL volumetric flask respectively, add 70 mL nanopure water, 5 mL Follin-Denis, 10 mL sodium carbonate solution then add nanopure water to volume and shake to mix. Set the timer and allow the test solution to complete the reaction at 30 °C for one hour. Analysis was conducted by using Spectrophotometry UV-Vis at ƛ 760 nm with 1 cm cell. Using the gallic acid absorbance and standard concentration, calculate two-variable linier regression equation. Calculation of total polyphenol (%) = [C/(M x m x L_2/L_1 x 1000)] x 100, in which C, concentration in mg/mL from standard curve, L_1 is total volume of the sample preparation (mL); L is the pippete volume of the sample preparation (mL); m is Index aridity of the sample (%) and M is the sample weight (g) [3].

RESULTS AND DISCUSSIONS

Material Characterization

Fresh spinach (*Amaranthus* sp.) is a dark green vegetable and is widely consumed as a good source of nutrition. Pulverizing process at a 1 part : 4 parts ratio of spinach (after blanching 80 °C for 15 minutes) to water showed difference in concentrations of total solids, total polyphenol and chlorophyl on pulp spinach and spinach filtrate, as displayed in Table 1.

TABLE 1. Characteristic of spinach as good source of antioxidant.

Kind of spinach	Composition		
	Total solids (%)	Total polyphenol (%)	Chlorophyl (mg/L)
Fresh spinach	18.34	0.2960	10.2642
Pulp spinach*	15.46	0.1276	0.876
Spinach filtrate**	1.34	0.1749	0.0375

Legend : *blanching result at 80 °C for 15 minutes, pulverizing at ratio 1 : 4; **via 80 mesh sieve; ***fermentation result using kombucha culture for 15 days; by kombucha culture at first time of fermentation (0 week).

Principle differences in pulverizing process was showed on spinach filtrate filtered through 80 mesh sieve with total solids 1.34 % and chlorophyl 0.0375 mg/L, respectively, smaller when compared with pulp spinach with total solids 15.46 % and chlorophyl 0.876 mg/L, respectively. This diffrence in result does not seem on total polyphenol, in which pulp spinach and spinach filtrate through 80 mesh sieve gave total polyphenol 0.1276 % and 0.1749 %, respectively, lower when compared with fresh spinach (0.2960%). Blanching process decreases possibility components concentration in spinach, besides pulverizing process (1 : 4 ratio) and filtration. The filtration through 80 mesh sieve showed that polyphenol compounds are associated with other components so that differences in pulp spinach and spinach filtrate are not wide. Dissolving components affect on total solids, although this matter is effected by solubility properties of polyphenol and chorophyl in water. It had been known that both kinds of components are easy dissolved in water [10]. Figures 3a, 3b and 3c represented subsequent fresh green spinach leaves, pulp spinach from pulverizing process and filtrate from sieving 80 mesh.

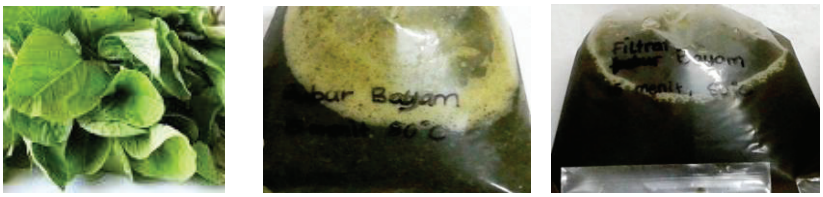

FIGURE 3. (a) Fresh green spinach (*Amaranthus* sp.) leaves, (b) pulp spinach as a result of pulverizing process from 1 part spinach (after blanching 80 °C for 15 minutes) and 4 part water (c) spinach filtrate sieved through 80 mesh.

Effect of fermentation process on biomass composition

Spinach fermentation using kombucha culture (25 % v/v) followed by adding sucrose (10 % w/v) on spinach extract at a 1 part : 4 parts ratio of spinach to water at initial fermentation (0 weeks) gave brighter and turbid suspension and seemed kombucha culture as gelatinoid fungi tissue, flat disc shape containing acetic acid bacteria (*Acetobacter xylinum*), yeast (*Saccharomyces cereviceae*, *Saccharomyces ludwigii*, *Saccharomyces bisporus*, *Zygosaccharomyces* sp.) and several khamir types (*Torolupsis* sp.) [1] floats on spinach extract. After 2 weeks of fermentasi of fermented spinach biomass seemed as suspension with degradation from green brownish, turbid yellowish and clear yellowish, as shown in Figure 4.

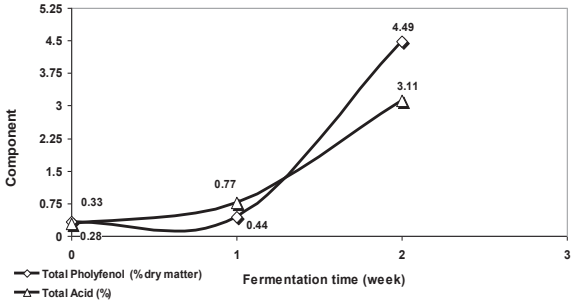

FIGURE 4. Effect of fermentation time on recovery of total acids and total polyphenol in biomass of fermented spinach by kombucha culture.

Its composition indicates occurrence of an increase of total acids and polyphenol. Total acids indicate ability of kombucha culture to ferment and convert biochemically substrate (sucrose) as good carbon source for its metabolism and is metabolite yielded by microbes (yeast and acetic acid bacteria) for running fermentation [1]. Total acids as organic acids (acetic acid, acetaldehyde) are produced through fungi hydrolysis from sucrose into glucose and fructose, and these into gluconic acid and acetic acid, and these substances are present in final fermented product. In this same condition, it occurs an increase polyphenol by long fermentation time, which is possibility caused by activity of katalase enzyme from *Candida tropicalis* as predominant factor of kombucha culture [2]. Phenolic compounds (phenolic acids and flavonoid) in spinach will be extracted by more much result under the best fermentation condition so that it increases its concentration expressed as total polyphenol. When compared with starting substances, spinach fermentation by using combucha culture increased total acids 90.84 % from starting fermentation (0 week) (0.285 % to 3.11 %) into 92.76 % to final fermentation (0.325 % to 4.49 %), respectively.

Long fermentation time increases reducing sugar and TPC and decreases total solids, as shown in Figure 5.

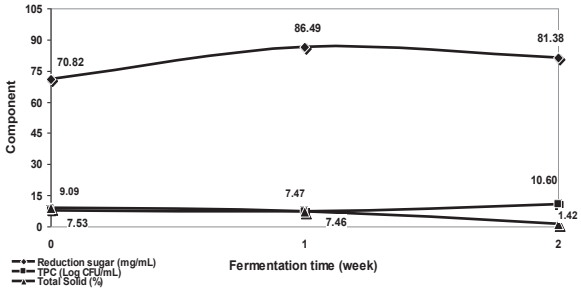

FIGURE 5. Effect of fermentation time on recovery of reducing sugar, TPC and total solids in biomass of fermented spinach by kombucha culture.

Reducing sugar in biomass is remained (sisa) sucrose after fermentation process, in which sucrose as a good source of carbon is converted biochemically into fructose and glucose. Further, bacterial components of kombucha culture, almost always including *Gluconacetobacter xylinus* (*G. xylinus*) and *Acetobacter xylinum* (*A.*

xylinum), degrades fructose and glucose into specific component (ethanol), and these into gluconic acid and organic acids (particularly acetic acid), that increases the acidity and limits ethanol concentration. Forming ethanol, acetaldehyde, and organic acids are influenced by quantity of starter, environmental conditions, and activity of invertase enzyme in forming metabolite. Besides, sucrose is used by *A. xylinum* bacteria to produce cellulose as 'nata' and float on the top medium surface [11]. Increasing reducing sugar concentration for 4 weeks of fermentation is caused by adding sucrose (15 % b/v) as energy source. Not all fructose and glucosea are utilized by microbes in producing organic acids, so that it is still remained reducing sugar in biomass. However, for the second weeks of termentation occurs a decrease of reducing sugar (81.38 mg/mL) due to high activity of microbes. This matter is relating with the growth rate of microbes, which decreases for the first week of fermentation (log 7.46 CFU/mL) and increases for the second weeks of fermentation (log 10.60 CFU/mL). On the whole fermentation processes, effect of fermentation rate will cause a change of components in biomass showed by lowering total solids. Because of both high concentrations of total acids and polyphenol, solubility of these components raise, but total solids become more and more low. When compared with initial substance, spinach fermentation by using kombucha culture increase reducing sugar 14.91% from starting fermentation (0 week) (70.82 %) to 2 weeks of fermentation (81.38 %) and TPC 40.77 % from starting fermentation (0 week) (log 7.53 CFU/mL) to 2 weeks of fermentation (log 10.60 CFU/mL), but drops total solids 84.39 % from starting fermentation (0 week) (9.09 %) to 2 weeks of fermentation (1.42 %). Figures 6a, 6b and 6c showed subsequently spinach fermented by kombucha culture in closed container at room temperature for initial fermentation (0 week), 1 week and 2 weeks.

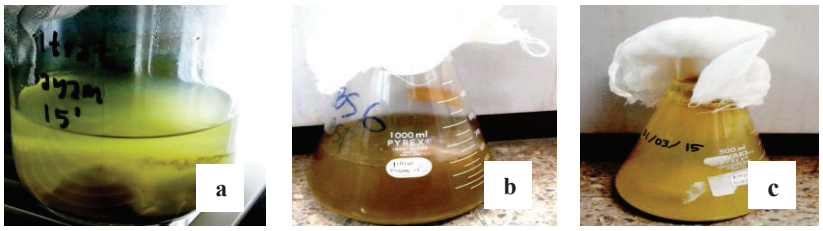

FIGURE 6. (a) Fermented spinach (*Amaranthus* sp.) by kombucha culture at room temperature for 0 week, (b) 1 week and (c) 2 weeks.

Identification of polyphenol extract of fermented spinach

From purifying fermented spinach through MF membrane (0.15 µm) fitted in dead-end SUFC under stirrer rotation 400 rpm, room temperature and pressure 40 psia for 30 minutes was get permeate (extract) followed by identifying polyphenol MW via LC-MS. Selection of permeate was carried out because components in biomass was predicted as polyphenol oligomer with particles size range from 0.001 to 0.01 µm, smaller than pores size of MF membrane (0.15 µm) [12]. Result of idensification showed that there is a difference in characteristic of oligomer shown from chromatogram for both kinds of biomass. Figure 7a displayed chromatogram gallic acid as standard for polyphenol. It is get 1 peak (T 2.8) with intensity 100 %, in which at mass spectra 111 – 784 mz from T 2.75 represents a domination of compound with MW 193.1289 Da. (100%) followed by compound with MW 363.2926 Da. (55 %) and 445.3647 Da. (35 %), as represented in Figure 7b.

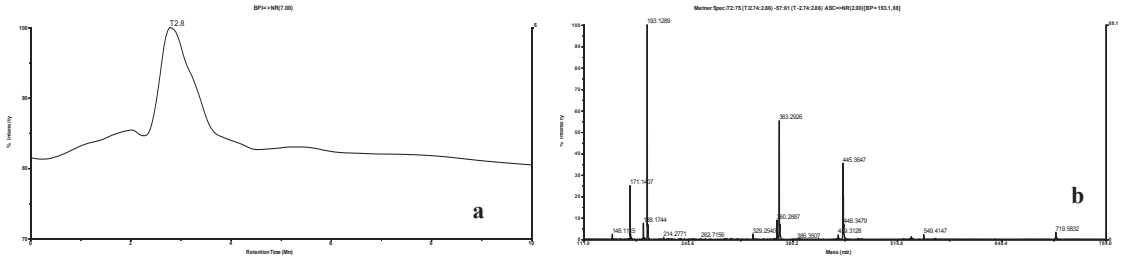

FIGURE 7. (a) Chromatogram of polyphenol monomer with retention time of 0 – 10 minutes at standard galic acid as polyphenol and (b) mass spectra between 111 – 784 mz from peak T2.7 at standard galic acids.

It had been known that MW of gallic acid as polyphenol ranges 193 Da. By using LC-MS is enabled a compound showing difference in MW, in which compounds are as M^+, M^+, Na^+, $2M^{++}$ or 2 M^{++}, Na^+. This matter is caused by its presence of ionization due to sensitivity of LC-MS instrument relating with eluent used. Operation condition of LC-MS is injection volume 2 μL, flow rate 0.05 mL/minute with methanol eluent (content 0.3 % acetic acid) [7].

Idensification on fermented spinach extract at starting fermentation (0 week) showed 1 dominant peak as T 3.0 with intensity 100 % for retention time between 0 and 20 minutes, as demonstrated in Figure 8a.

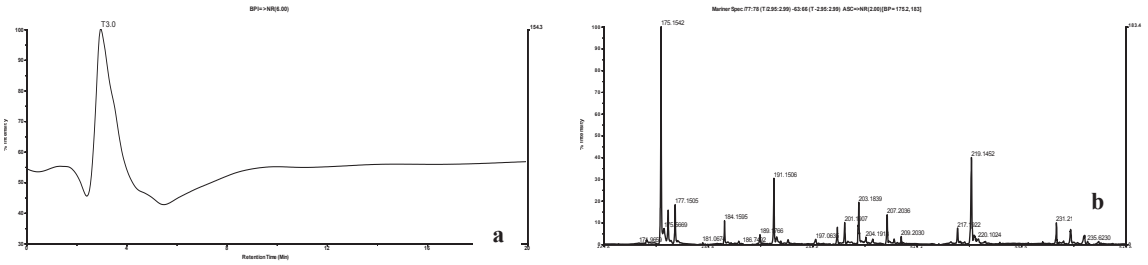

FIGURE 8. (a) Chromatogram of polyphenol monomer with retention time of 0 – 20 minutes, (b) mass spectra between 167 – 241 mz from peak T3.0 at spinach fermented by kombucha culture at initial fermentation (0 week).

Mass spectra 167–241 mz at T 3.0 in Figure 8b displayed possibility polyphenol compounds with MW 191.15, 191.55 and 193.16 Da. and relative intensities 30.29, 3.35 and 2.07% or in other words, total intensities are 35.71 % and 100 %. Identification as gallic acid was indicated by monomer with MW 193.1597 Da. and relative intensity 2.07%. Identification on fermented spinach extract for 1 week of fermentation indicates 2 dominant peak as T 2.7 and T 3.5 with intensities 100 % and 35 % for retention time between 0 and 10 minutes, as showed in Figure 9a. Mass spectra 170–199 mz at T 2.7 in Figure 9b predicts possibility polyphenol compound with MWs 191.25, 192.25, 193.27 and 194.29 Da. with intensities 13.51, 2.93, 6.34 and 5.19%, or in other words, total intensities are 27.97 % from 100 %. Identification as gallic acid is represented by monomer with MW 193.27 Da. and relative intensity 6.34 %. While, mass spectra 170 – 199 mz at T 3.5 in Figure 9b showed polyphenol compound with MWs 191.16, 191.26 and 191.92 Da. and intensities 58.50, 52.01 and 1.16 %, respectively. In other words, total intensities are 111,67 % from 35 % of intensity or 31.34 % at peak T 3.5, or the whole compounds are polyphenol. It is not found compound of gallic acid in this peak. For the whole idensifications, spinach extract fermented for 1 week contained polyphenol compounds with intensities 27.97 (T2.7) and 31.34 % or total 59.31 % from 7 kinds of polyphenol compounds with plyphenol oligomer MWs 191.25, 192.25, 193.27 and 194.29 Da. (T 2.7), and 191.16, 191.26 and 191.92 Da. (T 3.5). As gallic acid, it is only showed at T 2.7 with compound MW 193.27 Da. and intensity 6.34 %.

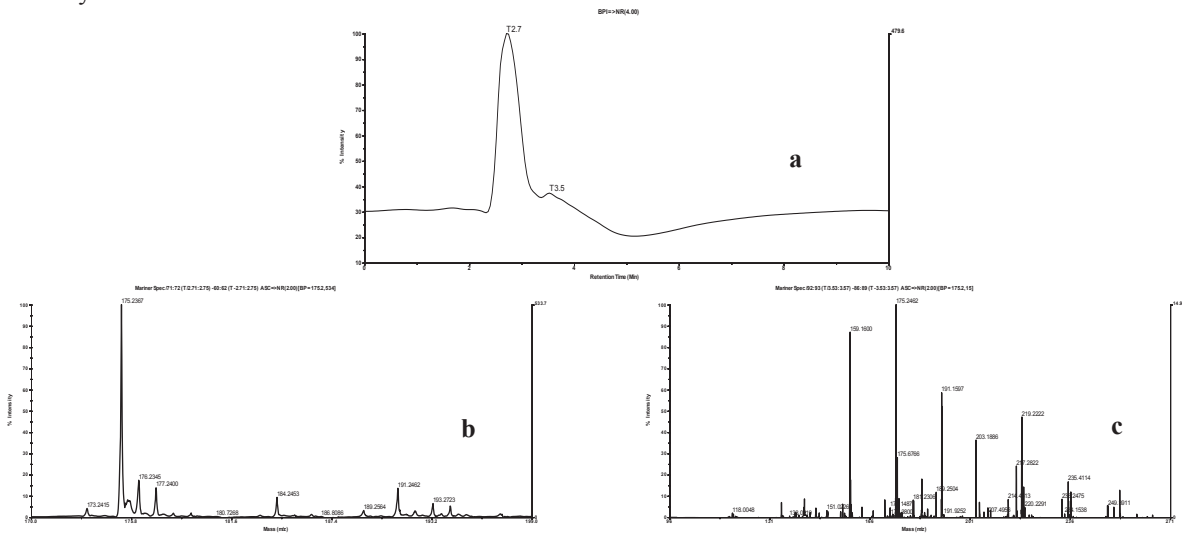

FIGURE 9. (a) Chromatogram of polyphenol monomer with retention time of 0 – 10 minutes for T2.7 and T3.5, (b) mass spectra between 170 – 199 mz from peak T2.7 and (c) at mass spectra between 111 – 784 mz from peak T3.5 for spinach fermented by kombucha culture for 1 week.

Identification on fermented spinach extract for 2 weeks of process showed 3 dominant peaks as T 2.5, T 2.9 and T 3.7 with intensity of 95, 100 and 45 % at retention time range from 0 to 10 minutes, as shown in Figure 10a. Mass spectra at T 2.5 displays polyphenol compounds with MW 191.17, 193.18 and 194.22 Da. and intensity 13.23, 8 and 1.86 %, respectively at retention time range from 183 – 201 minutes, as indicated in Figure 10b, or in other words, total intensity is 23.09 % of 100 %. Idensification of gallic acids is showed at monomer with MW 193.1871 Da. and relative intensity of 8 %. Mass spectra at T 2.86 demonstrates only 1 dominant polyphenol compound with MW 191.2866 Da.and intensity 3.27 % at retention time range from 162 – 216 minutes, as shown in Figure 10c, in other words, total intensity is 3.27 % of 100 %. Mass spectra at T 3.86 indicates only 1 dominant polyphenol compound with MW 191.19 Da. and intensity 0.61 % at retention time range from 162 to 216 minutes, as represented in Figure 10d or in other words, total intensity is 0.61 % of 100 %. It is not found gallic acid at both peaks. The whole idensifications, fermented spinach extract for 15 days gives 5 polyphenol compounds with total intensity 26.97 % with MW 191.1736, 193.1871 and 194.2170 Da. (T 2.5), 191.2866 Da. (T 2.86) and 191.19 Da. (T 3.86). As gallic acid is only shown at T 2.5 as compound MW of 193.27 Da. with intensity of 8 %.

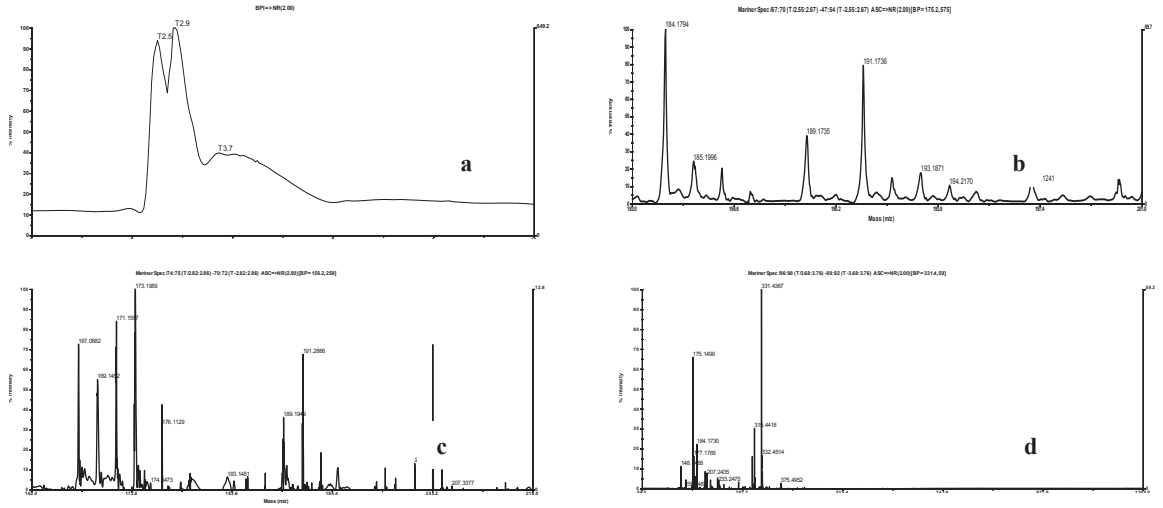

FIGURE 10. (a) Chromatogram of polyphenol monomer with retention time between 0 – 10 minutes and (b) mass spectra between 111 – 784 mz from T2.5, (c) mass spectra between 111 – 784 mz from T 2.86, and (d) mass spectra between 111 – 784 mz from T3.71 for spinach fermented for 2 weeks.

Based on total polyphenol concentration, the best fermentation condition to get fermented spinach as anti-oxidant was achieved for 2 weeks of fermentation process. Figures 11a, 11b and 11c showed fermented spinach at 2 week fermentation, permeate (extract) and retentate (concentrate) as a result of MF membrane (0.15 µm) fitted in SUFC.

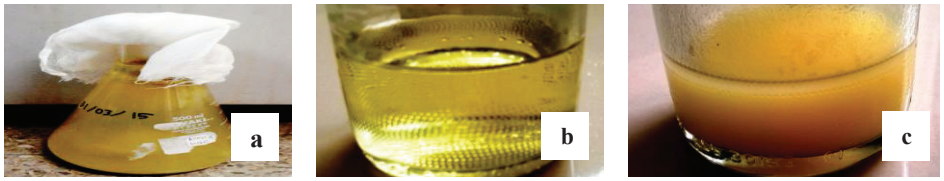

FIGURE 11. (a) Spinach fermented for 2 weeks used as a feed, (b) permeate (extract) and (c) retentate (concentrate) as a result of purification using MF membrane (0.15µm).

CONCLUSION

Fermentation of spinach by using kombucha culture increased the whole biomass components composition relating with long fermentation time. Based on recovery of total polyphenol, the best fermentation time was reached for 2 weeks under fixed process condition (kombucha culture concentration 25 % v/v, sucrose 10 % w/v, room temperature). In this process condition, it showed an increase of total polyphenol of 92.76 % from before

fermentation (0.325 % dry matter) to after fermentation (4.489 % dry matter). Based on standard gallic acid (MW 193.27 Da.), idensification of polyphenol oligomer showed that 2 weeks of fermentation yielded higher relative intensity (8 %) from 3 peaks when compared with 1 weeks of fermentation (6.34 %) from 2 peaks. In other words, spinach fermented for 2 weeks had stronger antioxidant property than when compared with spinach fermented for 1 weeks. Based on idensification of kind of polyphenol compound, 2 weeks of fermentation process generated 5 kinds of polyphenol compounds with total intensities 27.97 % giving MWs of 191.1736, 193.1871 and 194.2170 Da. (at T 2.5), 191.2866 (at T 2.86) and 191.19 (at T 3.86), while 1 week of fermentation process resulted 7 kinds of polyphenol compounds with total intensities 59.31 % giving MWs of 191.25, 192.25, 193.27 and 194.29 Da. (at T 2.7) and 191.16, 191.26 and 191.92 Da. (at T 3.5).

REFERENCES

1. Malbasa, R., Loncar, E. and Djuric, M. 2008. "Comparison of the products of Kombucha fermentation on sucrose and molasses", Journal of Food Chemistry 106: 1039 – 1045, (2008).
2. Jayabalan, R., Subathradevi, Marimuthu, S., Sathshkumar, M. And Swaminathan, K," Changes in free-radical scavenging ability of kombucha tea during fermentation". Food Chem. 2008, 109 (1) pp. 227 – 234 (2008).
3. Z. Liu, "New techniques for tea catechins extraction," in *International Training Workshop of Tea Science* (Hunan Agricultural University. China, 21 July-10 August 2006).
4. Anonymous. *Membrane Technology For Process Industry,(* http:www.pcims.com./ images/TP 105.5us.pdf; PCI Membrane System Inc. Milford, USA, 2005).
5. A. S. Michael,.*Handbook of Industrial Membrane Technology*, (Noyes Publications, Park Ridge, U. S. A., 1989)
6. D. Onggo, "General Principles in Electrospray Mass Spectrometry: A New Technique in Mass Spectral Analysis", Journal JMS **3(2)**, 115–131 (1998).
7. P. Eichhorn and T. P. Knepper, 2001"Electrospray ionization mass spectrometric studies on the amphoteric surfactant cocamidopropylbetaine", Journal of Mass Spectrometry**36**, 677 – 684 (2011).
8. AOA,. *Official Methods of Analysis*, (Association of Official Analytical Chemists, Washington, D. C, 1995)
9. Anonymous, *Catalogue and Product Information of Stirred Ultrafiltration Cell*, (Amicon Bioseparation, MILLIPORE, Bedford, U. S. A, 2002)
10. H. D. Belitz and W. Grosch, *Food Chemistry*, 2nd Edition (Springer-Verlag, Berlin Heidelberg, Germany, 2002).
11. Anonymous.. *Kombucha*, (Wikipedia Indonesia. Available from : http://en.wikipedia.org/wiki/Kombucha, accessed 10 March 2008).
12. Scott Keith.. *Handbook of Industrial Membranes,* 2nd. Edition, Elsevier Advanced Technology, Oxford, 763-768, (1998)