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Characterization, antioxidative and bifidogenic effects of polysaccharides from *Pleurotus eryngii* after heat treatments

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ABSTRACT

Polysaccharides were extracted from freeze-dried-, oven-dried- and boiling-treated *Pleurotus eryngii* (FDPEPS, ODPEPS and BTPEPS). Yield of FDPEPS and its total carbohydrates, total polysaccharides, reducing sugars, phenolics and protein content were higher than ODPEPS and BTPEPS. High performance liquid chromatography (HPLC) identified that FDPEPS, ODPEPS, and BTPEPS were composed of mannose (5.75%, 5.52%, 6.97%), glucose (88.90%, 89.31%, 87.68%) and galactose (5.34%, 5.17%, 5.35%). All three polysaccharides showed typical saccharic absorption bands in FT-IR. The FDPEPS showed the highest antioxidant activities in ferric reducing antioxidant power (FRAP), ABTS, superoxide anion and hydroxyl radical scavenging tests. Denser aggregates and larger serum pores were observed in confocal micrographs of soymilk added with ODPEPS. Viability of *Bifidobacterium longum* in soymilk added with polysaccharides was significantly higher ($p < 0.01$) than those without polysaccharides during fermentation. Heat treatments applied before extraction affected the properties, composition and microstructures of FDPEPS, ODPEPS and BTPEPS.

1. Introduction

Living organisms produce oxygen reactive species (ROS) and other free radicals continuously which may be related to diseases such as cancers, diabetes and atherosclerosis (Valko et al., 2007). Synthetic antioxidants, BHA and BHT, are widely applied in fat and oil based foods to prevent oxidative deterioration. However, the use of synthetic antioxidants is restricted as they are suspected to have toxic and possibly carcinogenic effects (Ito et al., 1986). Therefore, there have been increased interests to find natural antioxidants to help retard oxidative damages to human. Mushrooms, rich in polysaccharides, have been used as an important edible and medical resource for centuries (Ferreira, Barros, & Abreu, 2009). *Pleurotus eryngii* (*P. eryngii*) is one of the most important species of oyster mushrooms and contains high levels of polysaccharides, polyphenols, peptide, and dietary fiber (Mishra et al., 2013).

Probiotic bacteria are major components of intestinal microflora which are considered beneficial in controlling intestinal infections, lowering serum cholesterol levels, improving lactose utilization et al. *Bifidobacterium* is one of the predominant probiotic members of the intestinal microflora which is widely used in the preparation of dairy products in conjunction with *Lactobacillus acidophilus* (Shah, 2000). Asian populations have the lowest incidence of chronic diseases that have relatively high intake of soy-derived isoflavones. Soymilk (SM) is rich in various beneficial substances especially isoflavones, which have been fermented with different probiotics to improve its nutritional value (Otieno, Ashton, & Shah, 2006). While, there are only few reports about antioxidant activities and bifidogenic effects of *P. eryngii* polysaccharides especially those extracted from *P. eryngii* after heat treatments.

Identifying structures of *P. eryngii* polysaccharides is important to investigate its bioactivities and health benefits. However, the study on monosaccharide compositions of *P. eryngii* polysaccharides was rarely reported. Gas chromatograph (GC), high performance liquid chromatography (HPLC) and high performance capillary electrophoresis (HPCE) have been applied on polysaccharide structures and characteristics analysis according to various separation and detection method (Rohrer, Thayer, Weitzhandler, & Avdalovic, 1998). The HPLC method of pre-column derivatization has been widely used to simultaneous determination of neutral, acidic and basic carbohydrates. The reagent 1-phenyl-3-methyl-5-pyrazolone (PMP) which possesses strong UV absorbance at 245 nm is one of the popular labels that can react with reducing carbohydrates under mild conditions, requiring no acids catalyst and causing no desialylation and isomerization (Honda et al., 1989). Fourier-transform infrared spectra (FT-IR) has been widely used to identify vibrations of molecules and polar bonds between

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different atoms which can investigate glucosidic bonds and other functional groups in polysaccharides. Additionally, few publications report using scanning electron microscopy (SEM) to investigate polysaccharide structures while fixation and dehydration during SEM sample preparation will alter the native structures of polysaccharide. Confocal laser scanning microscopy (CLSM) has been generally used to observe microstructural changes without disturbing polysaccharides which can identify the localization of individual biopolymer phases of multicomponent biopolymer systems by staining each biopolymer with a fluorescent dye and exciting the dye using a laser light at a specific wavelength. Accordingly, individual distribution and conjugation of polysaccharide and protein can be visualized and compared using CLSM (Abhyankar, Mulvihill, Chaurin, & Auty, 2011; Xu, Chen, Wang, & Zhang, 2009).

The principal objective of this study was to extract polysaccharides from freeze-dried-, oven-dried- and boiling-treated *P. eryngii* (FDPEPS, ODPEPS and BTPEPS), and then to determine its ingredients composition, to identify its characteristics by HPLC, FT-IR and CLSM analysis, to examine its antioxidant activities *in vitro* and to test its bifidogenic effect on *Bifidobacterium longum* (*B. longum*) CSCC 5089 during soymilk fermentation.

2. Materials and methods

2.1. Chemicals and reagents

Arabinose, fucose, galactosamine, galactose, galacturonic acid, glucosamine, glucose, glucuronic acid, mannose, ribose, xylose, brilliant green, bovine serum albumin (BSA), Coomassie brilliant blue G-250, dinitrosalicylic acid, fluorescein isothiocyanate (FITC), Folin–Ciocalteau reagent, gallic acid, potassium ferricyanide, ferric chloride, ferrous sulphate, glycine, horseradish peroxidase, hydrogen peroxide (30%), L-cysteine-hydrochloride, phenol, pyrogallol, trichloroacetic acid, trifluoroacetic acid (TFA), trypsin, 1-phenyl-3-methyl-5-pyrazolone (PMP), 2-*tert*-butyl-4-methoxyphenol (BHA), 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic) acid (ABTS), 2,4,6-tripyridyl-s-triazine (TPTZ) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acetonitrile, chloroform and sodium dodecyl sulfate (SDS) were purchased from Merck Serono Co. Ltd. (Darmstadt, Germany). Dehydrated de Mann Rogosa Sharpe (MRS) broth was purchased from Becton, Dickinson and Company (Franklin Lakers, NJ, USA). Soy protein isolate (SPI) was purchased from The Solae Company (Shanghai, China).

2.2. P. eryngii preparation

One and a half kilogram of fresh *P. eryngii* purchased from a local market was cleaned to remove any residual compost, and cut in 3 mm thick slices. One third of the slices were freeze dried and the other one third of the slices was oven dried at 100 °C. The remaining one third of the slices was treated using boiling water as previously reported by Li and Shah (2013). All samples were lyophilized and milled to fine powders using an 80 mesh size screen and mixed thoroughly to obtain freeze-dried-, oven-dried- and boiling-treated *P. eryngii* (FDPE, ODPE and BTPE) powders. Powders were stored at −20 °C for further analyzed.

2.3. Polysaccharides extraction

Polysaccharides were extracted from *P. eryngii* powders using the method reported previously by Li and Shah (2014). Briefly, 20.00 g *P. eryngii* powders were extracted with 95% ethanol (1:30, w/v) for 24 h to remove impurities and small lipophilic molecules at 23 °C. Residues were extracted with hot water (1:30, w/v) at 95.0 °C for 3 h. Then supernatant was precipitated by the addition of a 3-fold volume of 80% ethanol at 4 °C for 24 h. The precipitate was separated and dissolved in 20 mL distilled water, added with 3 mL of 10.0 mg/mL of trypsin (pH 8.1) at 37 °C for 3 h to remove the proteins and mixed with 10% H_2O_2 (solution/H_2O_2, v/v) to destain the pigments. Then, mixtures were immersed in 90 °C water bath for 5 min to remove H_2O_2 and then cooled immediately to 23 °C. Lastly, the solution was added with a 3 volume of 80% ethanol and precipitated at 4 °C for 24 h. The precipitate was separated and washed with anhydrous ethanol and lyophilized to obtain freeze-dried-, oven-dried- and boiling-treated *P. eryngii* polysaccharides (FDPEPS, ODPEPS and BTPEPS). Polysaccharides were stored at −20 °C for further analyzed.

2.4. Polysaccharides properties determination

Total carbohydrates and reducing sugars contents were determined by the phenol–sulphuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) and method that reported by Miller (1956) with D-glucose as a standard, separately. Total polysaccharides were considered as the subtraction of reducing sugars from the total carbohydrates. Total phenolics content was determined by the Folin–Ciocalteu method (Li & Shah, 2013). Gallic acid was used as a standard and total phenolics content was expressed as microgram of gallic acid equivalents (GAE) per gram of dry weight (DW) polysaccharides. Protein content was determined by Bradford method (Bradford, 1976) with bovine serum albumin (BSA) as a standard.

2.5. FT-IR analysis

The FT-IR spectra of FDPEPS, ODPEPS and BTPEPS were obtained using a FT-IR spectrometer (IRAffinity-1, Shimadzu Corporation, Kyoto, Japan) to detect their functional groups. Briefly, 2 mg polysaccharides were ground with 20 mg KBr powder and then pressed into pellets for FT-IR measurement in the frequency range of 4000–400 cm^{-1} using 16 scans at a resolution of 4 cm^{-1}.

2.6. Polysaccharides hydrolysis

Briefly, 10 mg of polysaccharide sample was dissolved in 1 mL of 4 M TFA in a glass tube. The tube was sealed under a nitrogen atmosphere and kept at 110 °C for 3 h in an oven to hydrolyze the polysaccharide into component monosaccharides. The mixture was centrifuged at 1000*g* for 5 min after being cooled to 23 °C. Then 1 mL of methanol was added into the supernatant and evaporated to dryness by blowing of nitrogen stream under heating at 30 °C. Then the sample was dried twice more as above. The dried residue was dissolved in 1 mL distilled water for subsequent derivatization.

2.7. Standard solution preparation

Original standard solution (5.0 mM) was prepared by dissolving each standard monosaccharide in deionized water. Quantified standard solution was obtained by appropriate dilution of original standard solution with deionized water. Solution was filtered through a 0.45 μm filter and stored at 4 °C for further experiments.

2.8. Monosaccharide derivatization

The PMP derivatization of monosaccharides was carried out following the method reported by Honda et al. (1989) with modification. Briefly, 100 μL of hydrolyzed polysaccharide sample or monosaccharide standard solution was mixed with 100 μL of 0.6 M sodium hydroxide. Then, the mixture was added with 200 μL of 0.5 M methanolic solution of PMP and mixed thoroughly

by a vortex mixer. The whole mixture was kept at 70 °C for 1.5 h in an oven. The mixture was neutralized with 200 μL of 0.3 M hydrochloric acid after being cooled to 23 °C. Then, 1 mL of chloroform was added to the mixture and shaken vigorously. The chloroform layer was discarded and the extraction process was repeated five times. The aqueous layer was filtered through a 0.45 μm filter for HPLC analysis. The derivatization procedure of both hydrolysate and monosaccharide standard samples must be carried out under the same condition. Fucose was added as the internal standard in hydrolyzed polysaccharides at the beginning of derivatization.

2.9. HPLC analysis

The HPLC analysis of PMP-derived monosaccharides was carried out on a Shimadzu LC-2010A HPLC system equipped with a quaternary gradient pump unit, an UV–Vis detector (190–700 nm), an autosampler (0.1–100 μL) and a column oven (273–333 K). The analytical column was a Kromasil C18 HPLC column (4.6 mm i.d. × 250 mm, 5 μm, Phenomenex, Torrance, CA, USA). The PMP derivatives elution was performed with a mixture of 0.1 M phosphate buffer (pH 6.7) and acetonitrile in a ratio of 83:17 (v/v) with a flow rate of 1 mL/min at 30 °C. The UV absorbance of the effluent was set at 245 nm. The PMP derivatives were quantified by comparing their values of peak area to calibrated standard curves.

2.10. CLSM analysis

Structural features of all samples were investigated using a Carl Zeiss LSM710 NLO CLSM. The CLSM was equipped with an inverted microscope and Zeiss objective lenses (magnification 5×, 10× and 40×) were used. Digital image files were acquired in a tagged image file format at 1024 × 1024 pixel resolution. For CLSM observation, 10 mg/mL polysaccharides solution and soymilk with 0.5% polysaccharides were stained with fluorescein isothiocyanate (FITC, 1 mg/mL in ethanol solution) for 30 min. Briefly, 1 mL sample was mixed with 10 μL of FITC. After 30 min, 100 μL of stained sample were pipetted to a glass slide, covered and applied to CLSM immediately. The CLSM observation was carried out in a dark room and the excitation/emission wavelength of FITC were set at 488 nm/495–559 nm.

2.11. Cultures fermentation

The SM was made using soy protein isolate (SPI). Standard SM was prepared by adding 40 g of SPI to 1 L of distilled water, and then FDPE powder, ODPE powder, BTPE powder, FDPEPS, ODPEPS and BTPEPS were added into SM respectively (sample/SM, 1:200, w/v). After mixing, pH was adjusted to 6.7 using 1 M sodium hydroxide, and samples were autoclaved at 121 °C for 15 min. *B. longum* CSCC 5089, originally obtained from CSIRO Starter Culture Collection, was activated by growing successively in sterilized MRS broth containing 0.05% (w/v) L-cysteine-hydrochloride 3 times followed by growing successively in SM 3 times at 37 °C for 24 h, and then the 4th transfer into sterile sample cultures using an inoculum dose of 3% (v/v).

2.12. pH measurement and enumeration

Change in pH was measured at 0, 12, 24, 36, and 48 h during the fermentation using a pH meter (Model 250A, Science International Corporation, Hong Kong). Samples were tempered to 23 °C before pH measurement. For the enumeration of *B. longum*, 1 mL aliquots of SM were taken at 0, 12, 24, 36, and 48 h and serial dilutions were carried out in sterile 0.85% saline (NaCl/water, 0.85/100, w/v). Subsequent serial dilutions were vortexed for 30 s individually before pouring 50 μL into MRS agar (L-cysteine-hydrochloride/MRS agar solution, 1:2000, w/v) plates. Plates were incubated at 37 °C for 24 h in an anaerobic jar. Plates showing 25–250 colonies were counted and results were expressed as colony forming units (CFU) per milliliter of the inoculated sample.

2.13. ABTS radical scavenging activity

The ABTS radical scavenging activity was determined using the method reported by Arnao, Cano, and Acosta (2001) with some modifications. Briefly, 50 μL of ABTS liquid substrate system (Sigma) was mixed with 50 μL (10 μg/mL) horseradish peroxidase and 800 μL 50 mM Glycine–HCl Buffer (pH 4.5). After incubating at 23 °C for 20 min, 100 μL various concentrations (0.0, 0.5, 1.0, 2.0, 4.0, 8.0 mg/mL) of aqueous polysaccharides was added and incubated in tubes covered with aluminum foil at 23 °C for 10 min. Absorbance value was measured at 414 nm. The ABTS radical scavenging activity (RSA) was calculated by the equation:

$$\text{ABTS RSA}(\%) = [(A_0 - A_1)/A_0] \times 100$$

A_1 is the absorbance value of the solution with different concentrations of samples. A_0 is the absorbance value of the ABTS solution without samples. Fresh BHA and Vc solutions were used for calibration.

2.14. Ferric reducing antioxidant power (FRAP)

The FRAP assay was carried out using the method previously reported (Li & Shah, 2014). Briefly, reagents for FRAP were prepared by mixing acetate buffer (3.1 g sodium acetate and 20 mL acetic acid per liter, pH 3.6), a solution of 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3$ at 10:1:1 (v/v/v). The mixture was incubated at 37 °C for 10 min. Then 1.8 mL FRAP reagent (Fe^{3+}-TPTZ mixture) was added to 0.2 mL of aqueous polysaccharides at various concentrations (0.0, 0.5, 1.0, 2.0, 4.0, 8.0 mg/mL) and incubated in tubes covered with aluminum foil at 23 °C for 30 min. Absorbance value was measured at 593 nm. Fresh BHA and Vc solutions were used for calibration.

2.15. Superoxide anion radical scavenging activity

The activities of polysaccharides to scavenge superoxide anion radical were determined using the method previously reported (Li & Shah, 2014). Briefly, 0.7 mL of phosphate buffer (50 mM, pH 8.34) and 0.2 mL of aqueous polysaccharides at various concentrations (0.0, 0.5, 1.0, 2.0, 4.0, 8.0 mg/mL) were mixed and stood at 23 °C for 20 min, then 0.1 mL pyrogallol (3 mM) preheated to 23 °C was added. The mixture was mixed and the absorbance value was measured at 325 nm every 30 s for 5 min. Superoxide anion RSA was calculated using the following equation:

$$\text{Superoxide anion RSA}(\%) = [(\Delta A_0 - \Delta A_1)/\Delta A_0] \times 100$$

ΔA_1 is the absorbance value of the solution within per 30 s with different concentrations of samples. ΔA_0 is the absorbance value of the solution within per 30 s without samples. Fresh BHA and Vc solutions were used for calibration.

2.16. Hydroxyl radical scavenging activity

The activity of polysaccharides to scavenge hydroxyl radical was determined using the method previously reported (Li & Shah, 2014). Briefly, the reaction mixture containing 0.2 mL of 0.45 mM brilliant green, 0.5 mL of 0.5 mM $FeSO_4$, 0.5 mL of 3.0% H_2O_2, and 0.3 mL of aqueous polysaccharides at various concentrations (0.0, 0.5, 1.0, 2.0, 4.0, 8.0 mg/mL) were incubated in test tubes covered with aluminum foil at 23 °C for 30 min, and then centrifuged at 4000*g* for 5 min and the absorbance value was measured at 624 nm. Hydroxyl RSA was calculated by the following equation:

$$\text{Hydroxyl RSA}(\%) = [(A_s - A_0)/(A - A_0)] \times 100 \quad (1)$$

A_s is the absorbance value of the solution with different concentrations of samples. A_0 is the absorbance value of the solution in absence of the samples. A is the absorbance value of the solution in absence of the samples and Fenton Reaction System. Fresh BHA and Vc solutions were used for calibration.

2.17. Statistical analysis

Data reported are mean ± standard deviation for triplicate determinations of each sample. One-way analysis of variance (ANOVA) and Tukey's tests at 99% confidence intervals ($p < 0.01$) were performed using IBM SPSS Statistics 20.0.

3. Results and discussion

3.1. Polysaccharides extraction yields

The yield of FDPEPS was highest as compared with those of ODPEPS and BTPEPS which were in the order: FDPEPS (7.31 ± 0.15 g/100 g DW) > BTPEPS (7.16 ± 0.10 g/100 g DW) > ODPEPS (6.02 ± 0.12 g/100 g DW). The reasons for the decrease in the yield of BTPEPS could be losses due to solubilization of polysaccharides in hot water during boiling treatment (Li & Shah, 2013). Polysaccharides could be thermally degraded during heat treatment and proteins had different interactions with polysaccharides at high temperature (Doublier, Garnier, Renard, & Sanchez, 2000; Yang et al., 2015). Therefore, thermally degradation and interactions between denatured proteins and polysaccharides could be main reasons for the significantly decreased level ($p < 0.01$) of ODPEPS compared with those of BTPEPS and FDPEPS.

3.2. Polysaccharides properties determination

Composition of total carbohydrates, total polysaccharides, reducing sugars, total phenolics and protein in FDPEPS, ODPEPS and BTPEPS are shown in Table 1. The total carbohydrates content of FDPEPS (819.29 ± 27.32 mg/g) was the highest compared with those of ODPEPS (783.14 ± 44.15 mg/g) and BTPEPS (737.40 ± 26.60 mg/g). The reducing sugars content in FDPEPS (153.10 ± 0.87 mg/g) was significantly ($p < 0.01$) higher than those of ODPEPS (109.80 ± 0.56 mg/g) and BTPEPS (114.53 ± 2.25 mg/g). Additionally, the protein content in FDPEPS (20.54 ± 1.16 mg/g) was significantly higher than those of ODPEPS (9.61 ± 1.53 mg/g) and BTPEPS (7.82 ± 1.66 mg/g). Researchers reported that freeze drying could produce good dried products with high level retention of nutrient because of the absence of lipid water and low temperature required during the drying process (Ratti, 2001). Additionally, the Maillard reaction during drying or boiling process could reduce the nutritional ingredients (Mauron, 1980). These could be reasons for the highest contents of total carbohydrates, total polysaccharides, reducing sugars, total phenolics and protein in FDPEPS compared with those of ODPEPS and BTPEPS.

3.3. FT-IR spectral analysis

The FT-IR spectra of FDPEPS, ODPEPS and BTPEPS are shown in Fig. 1. The spectra of these three polysaccharides were almost identical to each other, indicating that they had almost the same chemical structure. The spectral range 820–900 cm^{-1} was the anomeric region because the vibrational bands for α- and β-configuration were well separated in this region (Xu et al., 2009). The absorption bands of FDPEPS (around 850 cm^{-1}) and ODPEPS (851 cm^{-1}) and BTPEPS (879 cm^{-1}) indicated that α-configuration existed in all three samples. Both FDPEPS (930 cm^{-1}) and ODPEPS (932 cm^{-1}) showed absorption bands around 930 cm^{-1} which was attributed to the C–O–C vibration of 3,6-anhydrogalactose (Maciel et al., 2008). There was no absorption band for BTPEPS around 930 cm^{-1} indicating the absence of 3,6-anhydrogalactose in BTPEPS. The absorption bands of FDPEPS (1242 cm^{-1}), ODPEPS (1242 cm^{-1}) and BTPEPS (1248 cm^{-1}) were derived from the stretching vibration of C–O, and the absorption bands of FDPEPS, ODPEPS and BTPEPS at 1418 cm^{-1} were assigned to the stretching deformation of an almost pure CH_2 group (Xu et al., 2009). Absorption bands at 3372/3370/3399 cm^{-1} and 1649/1649/1645 cm^{-1} were derived from stretching and bending vibration of O–H, which indicated hydroxyl groups existed in all three polysaccharides. Additionally, 2928/2930/2930 cm^{-1} were characteristic absorption bands for the stretching vibration of C–H (Prado-Fernández, Rodr ıguez-Vázquez, Tojo, & Andrade, 2003). Based on the FT-IR analysis, it may be concluded that FDPEPS, ODPEPS, and BTPEPS mainly exhibited α-configuration and contained carboxyl group.

3.4. HPLC analysis

The HPLC chromatograms of PMP-derived component monosaccharides released from FDPEPS and 11 standard monosaccharides are shown in Fig. 2. Additionally, the quantified monosaccharides released from FDPEPS, BTPEPS and ODPEPS are presented in Table 1. The monosaccharide composition of polysaccharides was identified by comparing the retention time with standards (Fig. 2, Table 1). Three monosaccharides, including mannose, glucose and galactose, were identified for polysaccharides from FDPEPS, BTPEPS and ODPEPS. Mannose, glucose and galactose in sample were calculated as microgram per gram DW (mg/g). There were no significant difference for the contents of mannose and galactose in FDPEPS (52 and 49 mg/g), BTPEPS (49 and 46 mg/g) and ODPEPS (53 and 41 mg/g). The content of glucose in FDPEPS (811 mg/g) was highest compared with those of BTPEPS (791 mg/g) and ODPEPS (667 mg/g). Additionally, percent contributions of mannose, glucose and galactose to the total monosaccharides content released from FDPEPS, BTPEPS and ODPEPS are

Table 1
Total carbohydrates, total polysaccharides, reducing sugars, phenolics and protein compositions and monosaccharide composition of FDPEPS, BTPEPS and ODPEPS. Values of total carbohydrates, total polysaccharides, reducing sugars and protein are expressed are microgram per gram dry weight (DW) polysaccharides, values of total phenolics are expressed as microgram of gallic acid equivalents (GAE) per gram of DW polysaccharides. The HPLC monosaccharide determination values are expressed as microgram per gram DW polysaccharides and mass content contribution to total monosaccharides of each sample is in parentheses. Values are expressed as mean ± standard deviation, $n = 3$. Values with no letters in common within each column are significantly different ($p < 0.01$).

	Property determination					HPLC monosaccharide composition		
	Total carbohydrates	Total polysaccharides	Reducing sugars	Total phenolics	Protein	Mannose	Glucose	Galactose
FDPEPS	819.29 ± 27.32^{a}	709.50 ± 27.29^{a}	153.10 ± 0.87^{a}	15.89 ± 1.17^{a}	20.54 ± 1.16^{a}	52.48 ± 3.90^{a} (5.75 ± 0.05%)	810.85 ± 11.43^{a} (88.90 ± 0.25%)	48.76 ± 3.58^{a} (5.34 ± 0.30%)
ODPEPS	783.14 ± 44.15^{ab}	629.99 ± 44.97^{b}	109.80 ± 0.56^{b}	8.98 ± 0.34^{b}	9.61 ± 1.53^{b}	52.86 ± 6.71^{a} (6.97 ± 1.15%)	667.45 ± 34.01^{b} (87.68 ± 1.06%)	40.75 ± 2.32^{a} (5.35 ± 0.10%)
BTPEPS	737.40 ± 26.60^{b}	622.85 ± 28.33^{b}	114.53 ± 2.25^{b}	13.11 ± 1.53^{a}	7.82 ± 1.66^{b}	48.95 ± 4.94^{a} (5.52 ± 0.41%)	791.38 ± 17.85^{a} (89.31 ± 0.43%)	45.82 ± 1.44^{a} (5.17 ± 0.02%)

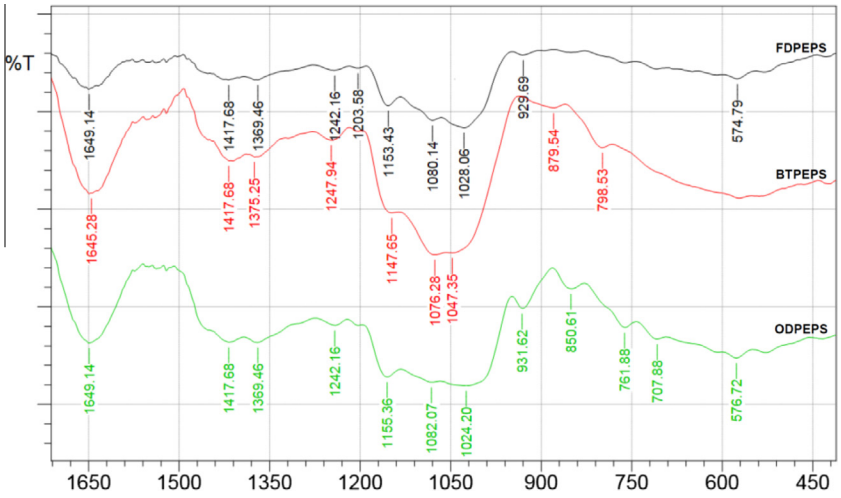

Fig. 1. The FT-IR spectrum of FDPEPS, ODPEPS and BTPEPS in the frequency range of 1700–400 cm^{-1}.

also presented in Table 1. Mannose, glucose and galactose were common found monosaccharides in mushrooms (Liu et al., 2015; Siu, Chen, & Wu, 2014). In our study, glucose was the predominant monosaccharide found in FDPEPS (88.90%), BTPEPS (89.31%) and ODPEPS (87.68%) which was consistent with the results reported by Liu et al. (2015).

3.5. CLSM analysis

Confocal laser scanning microscopy images of FDPEPS, ODPEPS, BTPEPS, SM + 0.5% FDPEPS, SM + 0.5% ODPEPS and SM + 0.5% BTPEPS are shown in Fig. 3. Micrograph of ODPEPS and SM + 0.5% ODPEPS showed obvious aggregates (arrow) compared with those of FDPEPS, BTPEPS, SM + 0.5% FDPEPS and SM + 0.5% BTPEPS. Devi, Liu, Hemar, Buckow, and Kasapis (2013) reported that gelatin could lead to obvious protein aggregation with 0.6% (w/w) gelatin in milk–gelatin mixture. Xanthan could lead to aggregation in skim milk powder solution (5%, w/w) and milk protein concentrate solution (5%, w/w) when 0.5% xanthan was added (Hemar, Tamehana, Munro, & Singh, 2001). The ODPEPS was extracted from oven dried (100 °C) *P. eryngii* in which Maillard reaction had occurred during the drying process. Those insoluble aggregates showed in micrographs of ODPEPS and BTPEPS could be Maillard-type conjugates that formed during 100 °C oven drying or boiling treatment (Jiménez-Castaño, Villamiel, & López-Fandiño, 2007). Also, attractive interactions between protein and polysaccharide could form soluble and/or insoluble complexes which may lead to coacervation or associative phase separation in solution (Schmitt, Sanchez, Desobry-Banon, & Hardy, 1998). From the above, the FDPEPS, ODPEPS and BTPEPS could interact with proteins in the soymilk to form conjugates and most of the conjugates were soluble in SM + 0.5% FDPEPS and SM + 0.5% BTPEPS, which showed as homogeneous structure of the matrix in micrographs. Micrograph also showed some insoluble conjugates formed in SM + 0.5% ODPEPS which led to denser aggregates and obvious serum pores (black area). Additionally, those insoluble aggregates showed in ODPEPS micrograph might accelerate the protein–polysaccharide aggregation after ODPEPS were added into soymilk, which accelerated the formation of dense aggregates and obvious serum pores in SM + 0.5% ODPEPS.

3.6. B. longum enumeration

Changes in viable counts of *B. longum* in different samples over 48 h of fermentation are shown in Fig. 4(A). In general, the total counts of *B. longum* increased in all samples during the fermentation, the maximum viable counts were found at 36 h in SM (7.90 ± 0.18 log CFU/mL), SM with FDPE powder (8.01 ± 0.24 log CFU/mL), SM with ODPE powder (7.95 ± 0.23 log CFU/mL) and SM with BTPE powder (7.99 ± 0.15 log CFU/mL), there was a slight decline in the counts at 48 h for those groups. The counts of *B. longum* in SM with FDPEPS, ODPEPS and BTPEPS increased with fermentation and the maximum numbers were found at 48 h which were in the order of FDPEPS (8.73 ± 0.45 log CFU/mL) > BTPEPS (8.71 ± 0.48 log CFU/mL) > ODPEPS (8.63 ± 0.33 log CFU/mL). Additionally, the counts of *B. longum* in SM with FDPEPS, ODPEPS and BTPEPS were significantly higher ($p < 0.01$) than those in SM, and SM containing FDPE, ODPE or BTPE powders throughout the entire fermentation period. Oven drying processing may have affected the original structure of the polysaccharides and caused irreversible changes to their initial properties in some ways (Bonvehi & Coll, 1993). Hence, ODPE powder had no significant stimulatory effect on the growth of *B. longum* compared with SM without additives. Our results indicated that polysaccharides extracted from *P. eryngii* after three different heat treatments had significant bifidogenic effects as they stimulated the growth of *B. longum* significantly ($p < 0.01$). Pham and Shah (2008) reported that supplementary lactose stimulated the growth of *B. longum* in SM as there is no lactose present in SM. In our study, the PEPSs could be a good bifidogenic factor as they provided carbohydrate and stimulated the growth of *B. longum* during 48 h fermentation. Compared with SM without additives and SM added with *P. eryngii* powders, additional polysaccharides in SM also kept the growth of *B. longum* after 36 h which supported that PEPSs possessed good bifidogenic effect. Confocal micrographs showed that insoluble aggregates formed in ODPEPS solution which might lead to fewer polysaccharides in culture medium during fermentation. Furthermore, it was more difficult for *B. longum* to decompose and utilize polysaccharides and proteins as there were denser and larger protein-polysaccharide aggregates in SM + 0.5% ODPEPS (Fig. 3), which could be another reason for lower viable counts of *B. longum* in SM + 0.5% ODPEPS.

3.7. pH Measurement

The pH changes in different cultures during fermentation are shown in Fig. 4(B). Generally, pH in SM with different PEPSs decreased significantly ($p < 0.01$) and faster than that of SM with different heat-treated *P. eryngii* powders. The decrease in pH during the first 12 h fermentation was maximum in BTPEPS (5.86) which was significantly lower ($p < 0.01$) than that of ODPEPS

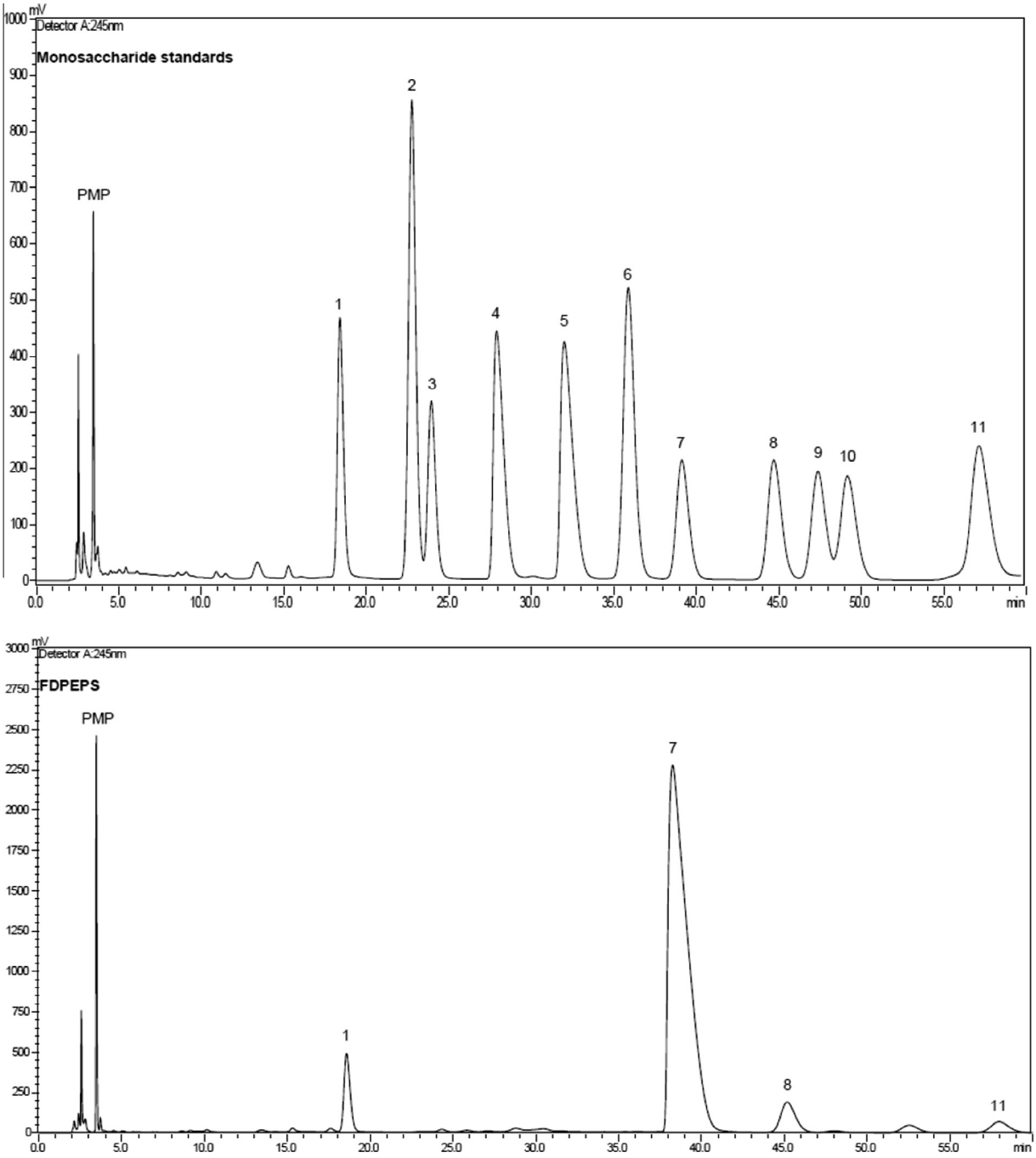

Fig. 2. The HPLC chromatograms of PMP derivatives of 11 standard monosaccharides and component monosaccharides released from FDPEPS. Peaks: (1) Mannose; (2) Glucosamine; (3) Ribose; (4) Glucuronic acid; (5) Galacturonic acid; (6) Galactosamine; (7) Glucose; (8) Galactose; (9) Xylose; (10) Arabinose; (11) Fucose (internal standard).

(5.93) while it was no significantly different with that of FDPEPS (5.88). Decrease in the pH was maximum in FDPE powder (6.26) which was lower ($p < 0.01$) than those of ODPE (6.30) and BTPE (6.29). After 48 h fermentation, the SM had the highest pH (5.69) while FDPEPS and BTPEPS had the lowest pH (5.31). During the entire fermentation period, pH of SM was significantly higher ($p < 0.01$) than those of all other samples, while pH of ODPEPS was significantly higher ($p < 0.01$) than those of FDPEPS and BTPEPS. Additionally, pH of SM added with polysaccharides was significantly higher ($p < 0.01$) than that of SM added with *P. eryngii* powders. Pham and Shah (2008) reported that the pH of SM decreased slightly from 6.80 to 6.29 during 24 h fermentation by *Lactobacillus* which was similar with results achieved in our study. Additionally, acetic and lactic acids were produced by *B. longum* during fermentation. Therefore, lower pH observed in PEPS samples supported the fact that PEPSs had good bifidogenic effect on *B. longum*.

3.8. ABTS radical scavenging activity

Antioxidant activities of FDPEPS, ODPEPS and BTPEPS determined using four *in vitro* assays are presented in Table 2. Values are expressed as μg BHA equivalents (BHAE)/100 mg DW and μg Vc equivalents (VcE)/100 mg DW. The EC_{50} is defined as the effec-

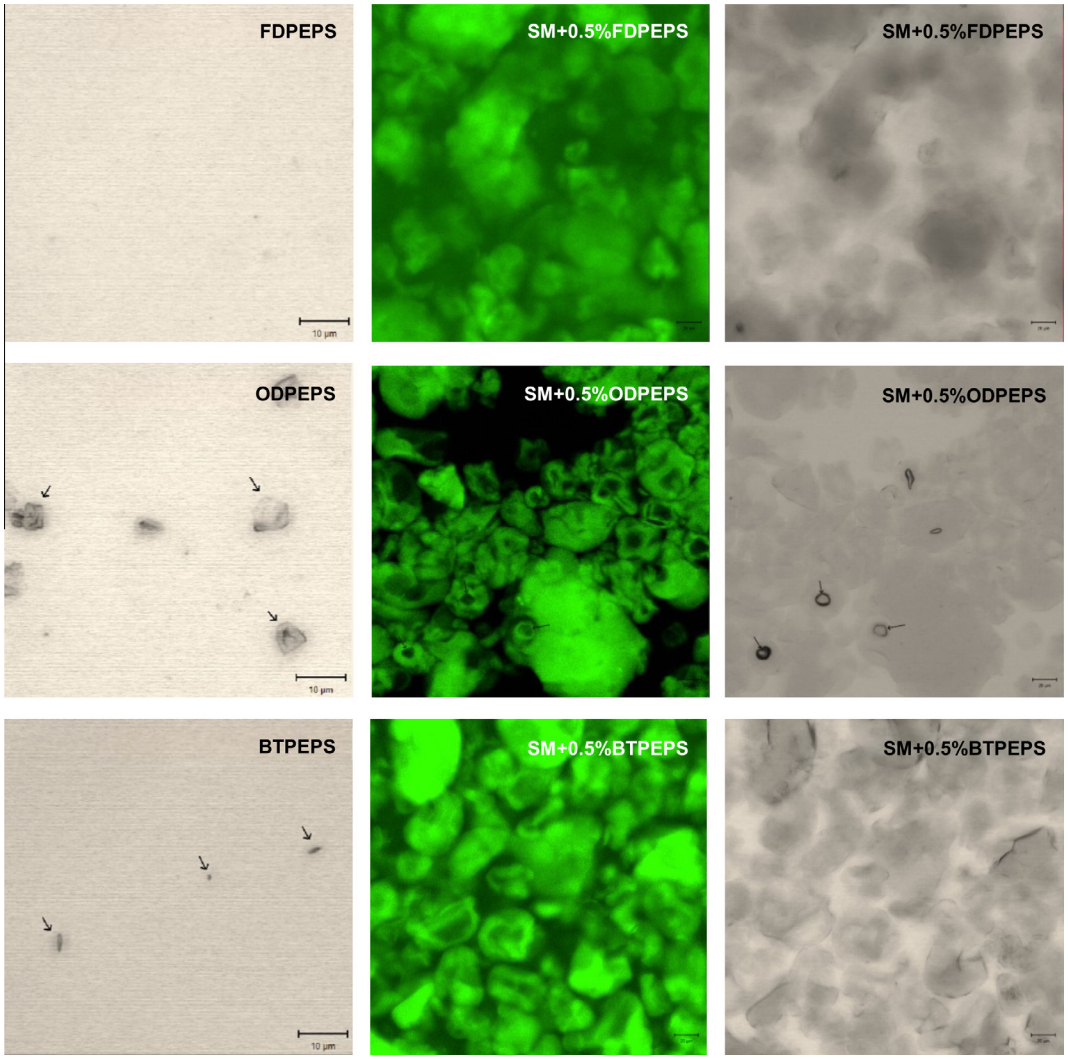

Fig. 3. Confocal laser scanning microscopy images of FDPEPS, ODPEPS, BTPEPS, SM + 0.5% FDPEPS, SM + 0.5% ODPEPS and SM + 0.5% BTPEPS. Scale bar for FDPEPS, ODPEPS and BTPEPS corresponds to 10 μm. Scale bar for SM + 0.5% FDPEPS, SM + 0.5% ODPEPS and SM + 0.5%BTPEPS corresponds to 20 μm.

tive concentration of samples which could scavenge 50% of radicals or the absorbance value is 0.5 in FRAP. The EC_{50} values are expressed as mg/mL. Scavenging activities of FDPEPS, ODPEPS and BTPEPS on ABTS radicals were significantly different ($p < 0.01$) which EC_{50} were in the order: FDPEPS > BTPEPS > ODPEPS (Table 2). The FDPEPS had the strongest scavenging activity (EC_{50} was 2.87 ± 0.44 mg/mL, 89.15 ± 0.20 μg BHAE/100 mg DW and 200.81 ± 0.48 μg VcE/100 mg DW) while ODPEPS had the lowest scavenging activity (EC_{50} was 6.93 ± 0.51 mg/mL, 57.42 ± 0.11 μg BHAE/100 mg DW and 123.65 ± 0.28 μg VcE/100 mg DW) (Table 2).

3.9. FRAP determination

Ferric reducing powers of FDPEPS, ODPEPS and BTPEPS were significantly different ($p < 0.01$) (Table 2). FDPEPS had the strongest reducing power (EC_{50} was 26.50 ± 4.02 mg/mL, 64.66 ± 0.42 μg BHAE/100 mg DW and 69.98 ± 0.45 μg VcE/100 mg DW) that was approximately 4-fold higher compared with that of ODPEPS (EC_{50} was above 100 mg/mL, 16.82 ± 0.13 μg BHAE/100 mg DW and 19.41 ± 0.13 μg VcE/100 mg DW). The reducing properties are generally associated with the presence of reductants which exert antioxidant activities by donating electrons to free radicals and break the free radical chain reaction (Huang, Ou, & Prior, 2005). Our results indicated that FDPEPS might donate more electrons or act as a more efficient electron donor in our FRAP assay.

3.10. Superoxide anion radical scavenging activity

The superoxide radical scavenging activities of three different polysaccharides had the same trend as scavenging activities on ABTS radical and ferric reducing antioxidant power. Overall, the FDPEPS had the strongest scavenging activity (EC_{50} was 8.59 ± 0.29 mg/mL, 56.70 ± 0.23 μg BHAE/100 mg DW and 84.81 ± 0.37 μg VcE/100 mg DW) which was approximately 2-fold higher compared with that of ODPEPS (EC_{50} was 55.09 ± 2.97 mg/mL, 27.92 ± 0.15 μg BHAE/100 mg DW and 37.88 ± 0.24 μg VcE/100 mg DW). The latter had the lowest scavenging activity among the three different polysaccharides.

3.11. Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activities of ODPEPS and BTPEPS were significantly lower ($p < 0.01$) than that of FDPEPS (Table 2). The FDPEPS had the strongest scavenging activity (EC_{50} was

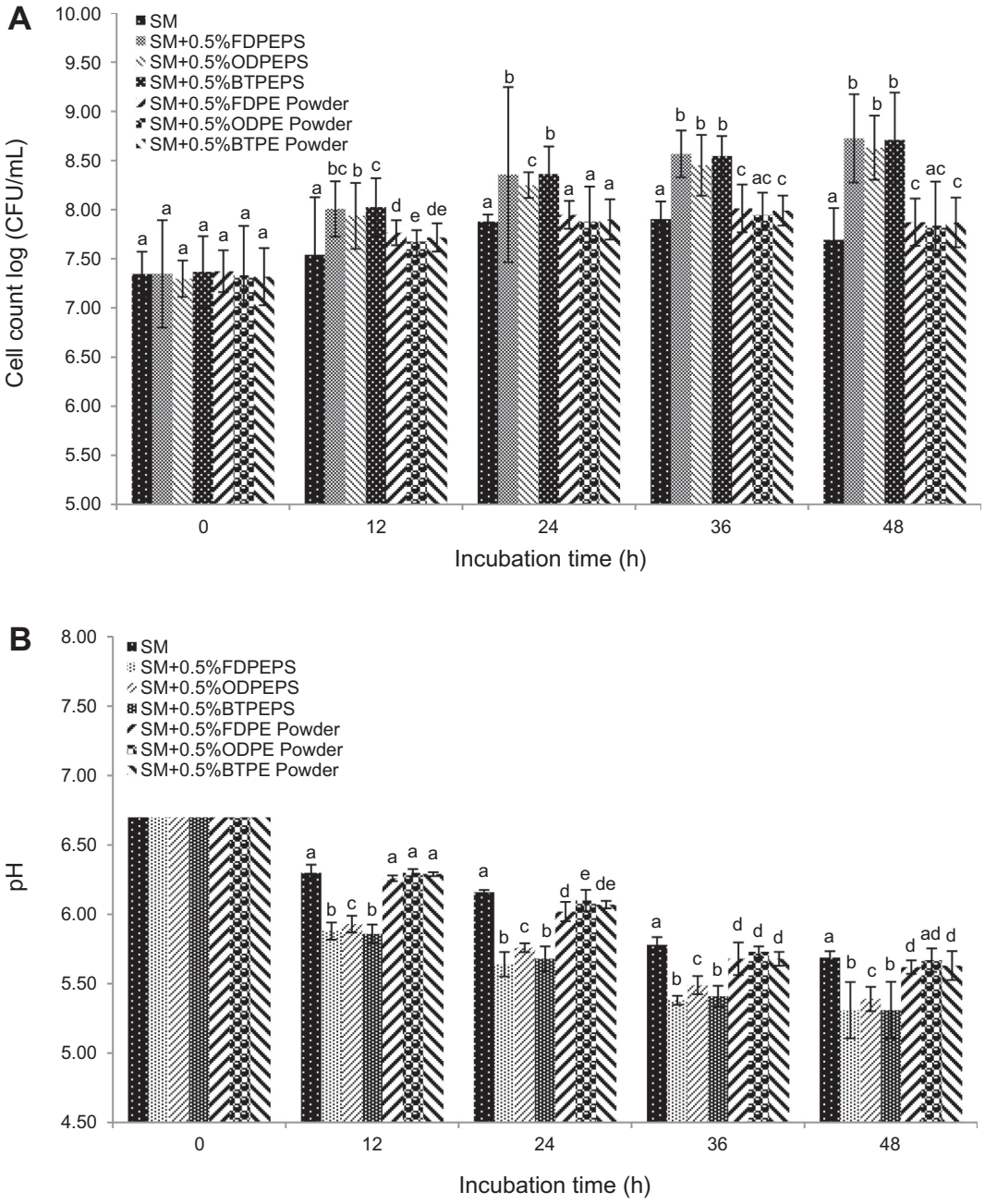

Fig. 4. Changes of viable counts of *B. longum* (A) and pH (B) in different samples incubated at 37 °C for 48 h. viability values are expressed as log CFU/mL. All values are expressed as mean ± standard deviation, $n = 3$. Values with no letters in common at each time (0, 12, 24, 36, and 48 h) are significantly different ($p < 0.01$).

Table 2
Antioxidant activities of FDPEPS, ODPEPS and BTPEPS obtained using four methods *in vitro*. Values are expressed as μg BHA equivalents/100 mg DW, μg Vc equivalents/100 mg DW and EC_{50} values are expressed as mg/mL (mean ± standard deviation, $n = 3$). Values with no letters in common within each row (EC_{50}, BHA equivalent and Vc equivalent respectively) are significantly different ($p < 0.01$).

	EC_{50}			BHA equivalents/100 mg DW			Vc equivalents/100 mg DW		
	FDPEPS	ODPEPS	BTPEPS	FDPEPS	ODPEPS	BTPEPS	FDPEPS	ODPEPS	BTPEPS
ABTS	2.87 ± 0.44^a	6.93 ± 0.51^b	3.48 ± 0.06^c	89.15 ± 0.20^a	57.42 ± 0.11^b	73.12 ± 0.13^c	200.81 ± 0.48^a	123.65 ± 0.28^b	161.82 ± 0.15^c
FRAP*	26.50 ± 4.02^a	>100.00	39.91 ± 3.40^b	64.66 ± 0.42^a	16.82 ± 0.13^b	44.42 ± 0.56^c	69.98 ± 0.45^a	19.41 ± 0.13^b	48.59 ± 0.60^c
Superoxide	8.59 ± 0.29^a	55.09 ± 2.97^b	22.97 ± 2.11^c	56.70 ± 0.23^a	27.92 ± 0.15^b	34.62 ± 0.04^c	84.81 ± 0.37^a	37.88 ± 0.24^b	48.80 ± 0.07^c
Hydroxyl	19.77 ± 2.10^a	37.22 ± 1.34^b	27.01 ± 0.99^c	56.14 ± 0.53^a	49.63 ± 0.32^b	50.60 ± 0.40^b	223.77 ± 1.40^a	202.90 ± 1.04^b	205.23 ± 1.29^b

* EC_{50} values for FRAP are presented as effective concentration at which the absorbance value is 0.5.

19.77 ± 2.10 mg/mL, 56.14 ± 0.53 μg BHAE/100 mg DW and 223.77 ± 1.40 μg VcE/100 mg DW) and ODPEPS had the lowest scavenging activity (EC_{50} was 37.22 ± 1.34 mg/mL, 49.63 ± 0.32 μg BHAE/100 mg DW and 202.90 ± 1.04 μg VcE/100 mg DW). The trend in hydroxyl radical scavenging activity presented in our study was similar to that reported by Fan, Li,

Deng, and Ai (2012) about three polysaccharides isolated from *Ganoderma lucidum* by hot air drying (GLP-H), vacuum drying (GLP-V) and vacuum freeze drying (GLP-F). Hydroxyl radical scavenging activities of GLP-F and GLP-V did not show any significant difference but were significantly higher ($p < 0.01$) than that of GLP-H (Fan et al., 2012).

Researchers reported that heat processing may cause irreversible modifications on the original structures of polysaccharides, which can lead to important changes in the proposed physiological and pharmacological properties of polysaccharides (Bonvehi & Coll, 1993; Femenia, Garcıa-Pascual, Simal, & Rosselló, 2003). From the above, the original structures of ODPEPS and BTPEPS have been affected during oven drying or boiling treatment and caused irreversible changes to polysaccharides initial properties in some ways. Thermal degradation and changes in properties of polysaccharides during heat treatment could be reasons for the significant decrease in radical scavenging activities and ferric reducing power of ODPEPS compared to FDPEPS and BTPEPS. Fig. 3 showed that there were obvious insoluble aggregates in ODPEPS solution which may lead to fewer functional groups of ODPEPS to act with radicals and ferric ions. Additionally, researches have shown that phenolic compounds exhibit strong antioxidant activities and therefore can be used as efficient antioxidants (Shahidi, Janitha, & Wanasundara, 1992). Therefore, the total phenolics reserved in FDPEPS, ODPEPS and BTPEPS may affect the antioxidant activities determination in our study.

4. Conclusions

The yield of FDPEPS was highest as compared with those of ODPEPS and BTPEPS. The FDPEPS had the highest contents of total carbohydrates (819.29 mg/g DW), total polysaccharides (709.50 mg/g DW), reducing sugars (153.10 mg/g DW), total phenolics (15.89 mg/g DW) and proteins (20.54 mg/g DW) compared with those of ODPEPS and BTPEPS. Antioxidant activities of FDPEPS as measured using four different methods were also the highest as compared to ODPEPS and BTPEPS, while the ODPEPS presented the weakest antioxidant activities as determined in four methods. Mannose, glucose and galactose were identified for polysaccharides from FDPEPS, BTPEPS and ODPEPS by HPLC pre-column PMP-derivatization method. Glucose was predominant in FDPEPS, ODPEPS and BTPEPS (88.90%, 87.68% and 89.31%). Characteristic absorption bands were observed in FT-IR which was almost identical to each other, indicating that FDPEPS, ODPEPS and BTPEPS have almost the same chemical structure. The confocal laser scanning microscopy images showed most of the insoluble aggregates in ODPEPS and also densest protein-polysaccharide conjugates in SM + 0.5% FDPEPS. The viable counts of *B. longum* in SM added with PEPSs were higher than those in SM without additives and SM added with *P. eryngii* powders during the entire fermentation period which indicated that polysaccharides had significant bifidogenic effect for *B. longum* compared with *P. eryngii* powders. The pH of SM added with PEPSs was lower than that of SM without additives and SM added with *P. eryngii* powders during the entire fermentation period which supported that PEPSs could be a good bifidogenic for *B. longum*.

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