Characterization and prebiotic activity *in vitro* of inulin-type fructan from *Codonopsis pilosula* roots

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Abstract

The inulin-type fructan was obtained by DEAE anion exchange chromatography from *C. pilosula* Nannf. var. *modesta* (Nannf.) L. T. Shen, after optimized extract condition, which was established by response surface methodology, designed using Box-Behnken factorial design and the optimum condition were: extracting 2.5 h with ratio of solvent to material 40 mL/g at 100 ^{o}C, twice. The maximum extraction yield was 20.6±0.2%. It was confirmed as β-(2-1) linkage fructan, with terminal glucose, and with a degree of polymerization of 2-17 (DP_{av}=6), shown by the results of methanolysis, methylation, nuclear magnetic resonance and molecular weight determination. The prebiotic activity was proven on account of stimulation effect on *Lactobacillus* and pH reduction of medium *in vitro*. The results indicated that the inulin from *C. pilosula* could be used as a potential natural source of prebiotics.

Keywords

Inulin-type fructan; Optimization; *Codonopsis pilosula*; Prebiotic activity

1 Introduction

Radix Codonopsis, the root of *Codonopsis pilosula* (Franch) Nannf, *C. pilosula* Nannf. var. *modesta* (Nannf.) L. T. Shen, and *C. tangshen* Oliv, is a traditional herbal medicine in Asian countries, and used as a replacement of ginseng (*Panax ginseng*) because of the similar pharmacological activities, such as immunological enhancement, delaying senility, lowering blood pressure and anti-ulcer (Fan & Hong, 2016; Tsai et al., 2013). The polysaccharide of *C. pilosula* is one of the major contributors to the biological activity (Feng et al., 2017; Guo, 2015), which may for instance inhibit intestinal mucositis and ulcerative colitis (Chen, 2016; Zhou et al., 2016), adjust the microecological imbalance (Chen, 2016; Wang, 2010), prevent tumor growth (Xin et al., 2012; Yang et al, 2013), modulate immune system functions (Zhang, 2015), and inflammation-promoting (Chu et al., 2016).

C. pilosula Nannf. var. *modesta* (Nannf.) L. T. Shen contains higher amount of polysaccharides than the other two *Codonopsis* species (Wang, Chen, Zou, Qin, & Zhu, 2016). At present, the optimum extraction condition of total polysaccharides (Zou, Chen, Yang, & Liu, 2011) structural elucidation and complement fixating activity of pectic polysaccharides have been investigated (Zou et al., 2014). However, there are few studies on neutral polysaccharides, especially inulin, being one of the microscopic identification indexes of identification of traditional Chinese medicine (TCM) powder to appraise the quality of *C. pilosula* (Yang, Li, Wei, Wang, & Li, 2011). Many studies represented the neutral polysaccharides of the other two species (Han, Cheng, & Chen, 2005; Ren, Zhang, Liu, & Sun, 2008; Zhang et al., 2005), only Liu et al. (2016) and Ye et al. (2005) indicated that the neutral polysaccharide from *C. pilosula* (Franch.) Nannf was fructan. There is no report on fructan from *C. pilosula* Nannf. var. *modesta* (Nannf.) L. T. Shen.

Inulin is composed of linear polymer of D-fructose with β-(2-1) fructosyl-fructose linkage, and terminal α-glucose. It was usually used as the replacement of sugar to persons suffering from obesity, diabetes and hyperlipidemia (Kelly, 2008; Tsurumaki et al., 2015; Yu et al., 2018). Besides, there are other pharmaceutical actions of inulin, such as intestinal flora modulation (Vandeputte et al., 2017), immunological

enhancement (Masanetz, Preißinger, Meyer, & Pfaffl, 2011; Vogt et al., 2015), prophylactic and therapeutic potential of inflammatory bowel diseases (IBD) (Leenen & Dieleman, 2007), antioxidant (Shang et al., 2018), anti-cancer and hepato-protective (Corrêa-Ferreira et al., 2017; Mensink, Frijlink, van der Voort Maarschalk, & Hinrichs, 2015). It could reach to the colon, not being enzymatic digested in the upper gastrointestinal tract, and is fermented to the short-chain fatty acids (SCFA) by the beneficial bacteria, ameliorating the dysfunction of the gastrointestinal tract, explaining the prebiotic activity (Shoaib et al., 2016; Wilson & Whelan, 2017; Yin, Fu, & Zhao, 2018). At the same time, the probiotics have the potential benefits of maintenance of mucosal nutrition and circulation, competition with harmful bacteria by colonization, production of antimicrobial substance, such as SCFA and bacteriocins, and modulation of the immune system, especially mucosal immunity (Moreno-Vilet et al., 2014; Meyer & Stassewolthuis, 2009), with potential therapy for IBD (Loh & Blaut, 2012).

As mentioned above, inulin-type fructan can selectively stimulate the enteric microorganism, especially *Lactobacillus* and *Bifidobacterium*, decreasing pH, restraining the colonization of harmful bacteria, preventing our body from pathogeny (Paßlack, Al-Samman, Vahjen, Männer, & Zentek, 2012). Considering the rare study of inulin from *C. pilosula*, the objective of the present study was to optimize the extraction and characterize the inulin, and evaluate the preliminary prebiotic activity *in vitro*.

2 Materials and Methods

2.1 Materials and reagents

The roots of *C. pilosula* were gathered from Jiuzhaigou County, Sichuan Province, China, and identified as *C. pilosula* Nannf. var. *modesta* (Nannf.) L. T. Shen by Yuan-feng Zou of Sichuan Agricultural University. A specimen (NO.20161015) is deposited in the College of Veterinary Medicine, Sichuan Agricultural University.

The MRS medium (HB0384-1), peptone (HB8276), tryptone (HB8270) were purchased from Hopebio Biotechnology Co., Ltd (Qingdao, China); the yeast extract powder (JM-500) was purchased from Biotopped Science and Technology Co., Ltd

(Beijing, China); the McIntosh Turbidimetric tube (G60346) was obtained from Wenzhou Kangtai Biotechnology Co., Ltd (Zhejiang, China); the standard fructooligosaccharide (QHT-FOS-P95S) and inulin (Orafti®HP) were purchased from Quantum Hi-Tech Biological Co., Ltd (Jiangmen, China) and Beneo-Orafti (Belgium), respectively.

The standard of fructose (Fru) and glucose (Glc) were purchased from Solarbio (Beijing, China). All other chemicals, such as phenol, sulfuric acid, acetone, boric acid, glycerin, etc., were of analytical grade, obtained from the Chengdu Kelong chemical factory (Chengdu, China).

2.2 Extraction and determination of fructan from *C. pilosula*

Two hundred gram of *C. pilosula* were dried in drying oven (DHG-9420A, Yi-heng technology Co., Ltd, shanghai, China) at 50 ºC (183.54 g) and pulverized to a fine powder by a mechanical grinder, then passed through 0.25 mm mesh. After extraction by refluxing 96% ethanol to remove low molecular weight and lipophilic compounds, the residue (175.80 g) was dried for further studies. Dried material (1.0 g) was extracted with distilled water with different extraction conditions. The aqueous extracts were mixed, adding distilled water to 100 mL, then the samples were determined for fructan concentration by the phenol-acetone-boric acid reagent (PABR) assay, which was described by Boratyński (1984), modified by Chaplin (1994), with high sensitivity (0.1-9 μg fructose in 100 μL) and selectivity (<1% from non-ketose carbohydrate) (Inngjerdingena et al., 2012). The extraction yield of fructan was calculated according to the ratio of the amount of fructan (g) to the original material (g).

2.3 Design of extraction conditions

2.3.1 Single-factor experiments

The optimum extraction condition of *C. pilosula* fructan was measured by single-factor experiments and response surface method (RSM); details were as follows: the single-factor experiment was executed in a designed extraction temperature (range from 50 to 100 ºC), ratio of solution to material (range from 10 to 60 mL/g) and extraction time (range from 0.5 to 3 h) (Apolinário et al., 2014; Kang et

al., 2018), keeping only one factor constant, with 1.0 g of *C. pilosula* defatted root powder for each experiment, each group in triplicate. Immediately after extraction, the yield of fructan present in *C. pilosula* defatted root powder was determined with the method described above.

2.3.2 Optimization of extraction conditions by BBD

At the base of single-factor experiment, a Box-Behnken factorial design (BBD), with three-level-three-factors, was employed (Table 1). These factors are mentioned above, the extraction temperature (X_1), the ratio of solvent to material (X_2) and extraction time (X_3), were designed using SAS. JMP. 13.0 software (Statistical analysis system, USA).

The variables were coded according to

$$X_i = \frac{x_i - x_0}{\Delta x} \quad (1)$$

where X_i is the coded value of the variable x_i, x_0 is the value of x_i at the central point, and Δx is the amplitude of variation. The result were analyzed and fitted to a second-order polynomial model:

$$Y_2 = A_0 + \sum_{i=1}^{3} A_i X_i + \sum_{i=1}^{3} A_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} A_{ij} X_i X_j \quad (2)$$

Where Y_2 is response variable (the extraction yield of fructan); A_0, A_i, A_{ii} and A_{ij} are intercept, linear, quadratic and interaction coefficients of X_1, X_2 and X_3, respectively; X_i , X_j are the coded independent variables; and the terms of X_i^2 represent the quadratic terms. Analyses of variance were evaluated by ANOVA procedure, and the fitness of this predictive model was performed by the coefficient of determination R^2 and the adjusted-R^2, then checking the statistical significance and regression coefficients using F-test at a probability (p) of 0.01 or 0.05.

Table 1 Central composite design and the extraction yield of fructan.

Runs	Independent variables						Extraction Yield/%
	Coded level			Uncoded level			
	X_1	X_2	X_3	x_1	x_2	x_3	
1	0	1	-1	90	50	1.5	19.0

2	0	-1	-1	90	30	1.5	17.1
3	-1	0	1	80	40	2.5	18.3
4	0	0	0	90	40	2.0	18.9
5	-1	-1	0	80	30	2.0	17.6
6	-1	0	-1	80	40	1.5	17.0
7	0	1	1	90	50	2.5	19.8
8	0	0	0	90	40	2.0	19.3
9	1	0	1	100	40	2.5	20.4
10	0	-1	1	90	30	2.5	19.4
11	1	-1	0	100	30	2.0	18.8
12	1	0	-1	100	40	1.5	18.5
13	1	1	0	100	50	2.0	19.3
14	0	0	0	90	40	2.0	19.5
15	-1	1	0	80	50	2.0	18.5

2.4 The purification of fructan from *C. pilosula*

The fructan was prepared from 100 g *C. pilosula* using the optimum extraction condition obtained in 2.3. The crude extracts were concentrated in a rotary evaporator at 80 rpm, 50◦C, 0.09 MPa (RE-3000, Yarong Biochemical Instruments Factory, Shanghai, China) and precipitated by 3-folds volume of ethanol 96% with respect to concentrated extracts, according to Li et al. (2017) and Caleffi et al. (2015). The precipitation was washed with ethanol and acetone, frozen at -80◦C, then lyophilized at -55◦C using a freeze dryer (TsiStar LycQuest), and named CPP (crude polysaccharides of *C. pilosula*). This was dissolved with 20 mL distilled water, filtered through 0.45 μm filter, and purified by DEAE-Sepharose (Fast Flow, FF) column (50mm×40cm, Beijing Rui Da Heng Hui Science Technology Development Co., Ltd., Beijing, China), an anion-exchange chromatography medium used commonly in fructan separation from other polysaccharides and non-carbohydrate compounds (Apolinário et al., 2014). The neutral fraction was eluted with 1.5-fold column volume (approximately 1 L) distilled water at the speed of 2 mL/min, combining the eluate until no sugar was detected by PABR method. Finally, the eluate was concentrated and lyophilized, nominated as CPPF (the fructan of polysaccharides from *C. picosula*).

2.5 Chemical compositions and linkage determination

The CPPF was subjected to methanolysis with 3 M hydrochloric acids in anhydrous

methanol for 24 h at 80◦C, obtain the methylglcosides. Then the monosaccharide composition was determined by gas chromatography (GC) after derivatization by hexamethyl disilazane (HMDS) and trimethylchlorsilane (TMS) reaction (Austarheim et al., 2012; Barsett, Paulsen, & Habte, 1992; Zou et al., 2014). The mannitol was added to the samples as the internal standard. Additionally, the presence of Fru was tested with the Urea-HCl colorimetric method (Dedonder, 1952).

The glycisidic linkages were determined by methylation. The carrier gas was Helium (pressure control: 80 kPa). The relative amount of each type of linkage was determined based on the area of each compound and related to the molecular weight of each compound (Austarheim et al., 2012; Zou et al., 2014).

2.6 The molecular weight determination

The solution of the CPPF in 0.1 mol/L sodium nitrate (5.8 mg/mL) was applied to a Viscotek TDAmax system (Malvern, UK) equipped with a pair of gel-filtration chromatographic column (7.8 mm*300 mm, Viscotek A6000M, General Mixed, Malvern, England) under a constant flow (1.0 mL/min) of 0.1 mol/L sodium nitrate at 30 ℃. The injection volume was 100 μL, and the eluate was monitored by Viscotek 270max detection system (differential viscometer detector and double-angle (right angle/low angle) light scattering detectors) combined with refractive index (RI) detector (Viscotek VE 3580/ Viscotek 270DUAL, Malvern, England). Gel permeation chromatography (GPC) analysis was performed using OmniSEC 5.0 software. The polyethylene oxide (PEO, *Mw*=18670 Da, 5 mg/mL, obtained from Sigma-Aldrich, 1546853) was used as standard.

2.7 The NMR spectroscopy

The ^{1}H NMR, ^{13}C NMR and DEPT spectra of CPPF were recorded on a Bruker spectrometer (600 MHz) after deuterium exchanged three times by freeze-drying in D_2O (10 mg/mL) and then performed on a Bruker AV600 instrument (Bruker, Rheinstetten, Germany) at 25 ^{o}C, labeling these peaks by MestReNova software (Version 6.0.2-5475, 2009, Mestrelab Research S.L., Spain).

2.8 Prebiotic effect

2.8.1 *Lactobacillus* bacterial strains

The *Lactobacillus buchneri* (BSS1, CCTCC No. AB 2016284), *L. johnsonii* (BS15, CCTCC: M 2013663), *L. plantarum* (BS10, CCTCC:M 2012487), *L. plantarum* (BSGP201683, CCTCC: M2016425) and *L. rhamnosus* GG (LGG, ATCC53103) were gifts from professor Xue-Qin Ni of Animal Microecology Institute, College of Veterinary Medicine, Sichuan Agricultural University, China; *L. johnsonii* (Hjg8, ATCC 33200) was a gift from Dr. Bing-zhao Zhang of Shenzhen Institutes of Advanced Technology, Chinese Academy of Science, China. They were stored at -80 ^{o}C in MRS medium, with 20% glycerin.

2.8.2 Bacterial growth

The basal (10 g tryptone, 10 g peptone, 5 g yeast extract, 1 mL of Tween 80, 0.5 g L-cysteine hydrochloride, 1 g/L carbohydrate source and 1 L of distilled water, pH 6.5) and MRS medium were autoclaved at 121 ^{o}C for 20 min. The CPPF was used as carbon source after filtered through sterile 0.22 μm filter. The P95s (96.1% fructo-oligosaccharides, DP_n 2-9, with 2.7% glucose, fructose and sucrose, product of the partial enzymatic hydrolysis of chicory inulin) and Orafti®HP (99.8% inulin, DP_{av} ≥23, with 0.2% glucose, fructose and sucrose), commercially available prebiotic, were used for comparison with CPPF; and the medium without carbohydrate was used as negative control (Caleffi et al., 2015; Lopes et al., 2017; Lopes et al., 2015; Li et al., 2015).

These six strains of lactobacilli were incubated in the 50 mL MRS medium at 37 ^{o}C overnight in anaerobic chamber (Thermo Scientific 1029, in 85% N_2, 10% H_2, 5% CO_2), then centrifuged 3500 rpm, 10 min, and resuspended in saline and basal medium, successively, to remove the carbon source. Finally, they were resuspended with basal medium containing these three different carbon sources above (the CPPF and two commercially available prebiotic P95s, Orafti®HP), at a concentration of 10^7-10^8 CFU/mL (Lopes et al., 2017; Lopes et al., 2015), after adjusted by McIntosh Turbidimetric tube. 5 milliliter bacterial suspensions were divided in test tubes, and then incubated for 0 and 24 h. All test tubes were set in triplicate.

Two hundred microliter of the basal medium was added to the 96-wells plates and the density of bacteria were measured at the wavelength of 600 nm (A_{600}) using

Multiscan Spectrum (Thermo Scientific, Varioskan Flash) after incubated for 0 h and 24 h. The bacterial growth was evidenced as the increment in A_{600} (ΔA_{600}) during 24 h of incubation in anaerobic chamber.

After 24 h of incubation, the pH was measured by pH meter (A115200, Lichen Instrument technology Co. Ltd., Hunan, China) after removing bacteria by centrifuging at 4000 rpm for 20 min. Each tube was tested three times, triplicate each time, making sure high accuracy and precision.

2.9 Statistical analysis

All data obtained above were analyzed by IBM SPSS statistic software (Version 20.0, USA) including the one-way analysis of variance with Duncan's test, presented as the means ± standard deviation (SD). The difference between groups were evaluated at statistically significant level of $p<0.05$.

3 Results and discussion

3.1 Effects of temperature, S/M ratio and time on extraction yield of fructan from *C. pilosula*.

The temperature was set at 50, 60, 70, 80, 90, 100 °C, at the S/M ratio of 30 mL/g, extracting time 1.5 h, twice. It was observed that the content of fructan increased significantly ($p < 0.05$) with the rise of temperature, close to a liner relation. The highest yield was 20.5% at 100°C (Fig.1A). Since the boiling point of water is 100°C, the range of extraction temperature in BBD was from 80 to 100°C, and the central point was 90 °C.

The S/M ratio was changed from 10 to 60 mL/g, with the gradient of 10 mL/g, extracting twice at 100°C and for 1.5 h each time. The results showed that the fructan yield increased significantly ($p < 0.05$) following the rising of S/M ratio at 10-40 mL/g (Fig.1B), while the yield of fructan increased only 0.16% from 40 mL/g to 60 mL/g (no significant difference). Maybe it was because most of the fructan was extracted from *C. pilosula* at the S/M ratio of 40 mL/g, so that the yield of fructan did not increase with the S/M ratio rising from 40 mL/g to 60 mL/g. Thus, to minimize electricity and time costs for further aqueous solutions concentration, the S/M ratio range of 30–50 mL/g was considered for use in further BBD experiments, and the

central point was 40 mL/g.

The extraction time was set from 0.5 h to 3 h, at the S/M ratio of 30 mL/g, extracted twice at 100°C. The results showed that the yield of fructan increased significantly ($p < 0.05$) when extracted from 0.5-2 h, and decreased 0.21-0.38% with a prolonged extraction time (no significant difference) (Fig.1C), indicated that extraction time of 1.5 to 2.5 h was to be used for further BBD experiments, with central point of 2.0 h.

Thus, the three factors three levels center design using the method of BBD were: extraction temperature 80, 90, 100°C; S/M ratio 30, 40, 50 mL/g; extraction time 1.5, 2, 2.5 h.

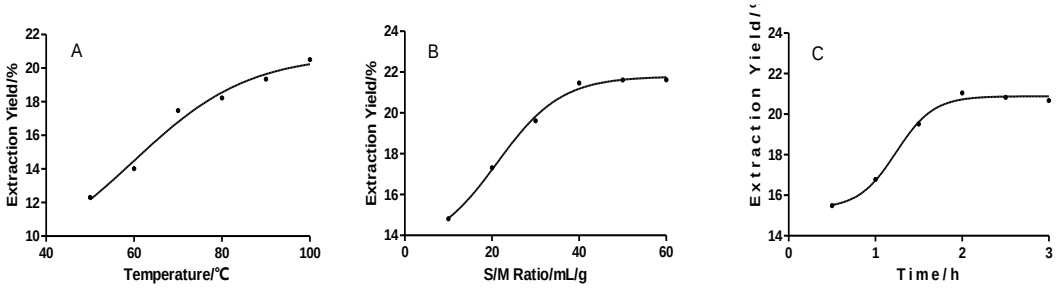

Fig.1. Effect of different extraction parameters on the yield of fructan from *C. pilosula*. A, extraction temperature (the S/M ratio and extraction time were fixed at 30 mL/g and 1.5 h, respectively); B, S/M ratio (the extraction temperature and time were fixed at 100 °C and 1.5 h, respectively); C, extraction time (the extraction temperature and S/M ratio were fixed at 100 °C and 30 mL/g, respectively). Values are the means of fructan yield (n=3).

It is worth mentioning that many previous studies have extracted inulin or fructo-oligosaccharides approximately at the temperature of 80-85°C from chicory roots, the globe artichoke, *Taraxacum kok-saghyz* roots and *Helianthus tuberosus* L. tubers (Apolinário et al., 2014; Hahn et al., 2016). The extraction of inulin with boiling water was frequently used, such as from *Artemisia japonica* (Li et al., 2017) and *Pfaffia glomerata* (Spreng) pedersen roots (Caleffi et al., 2015). Nevertheless, the optimal extraction condition was not indicated in their studies. Rubel et al. (2017) optimized the extraction of inulin from *Jerusalem artichoke* tubers, which was extracted at 76 ℃, employing a solid:solvent ratio of 1:16 for 90 min. Banerjee, Chowdhury, and Bhattacharya (2017) demonstrated that the optimal conditions of

292 inulin from *Pennisetum glaucum* were 70 ℃, HCl concentration of 0.8 M and heating
293 period of 60 min. In our study, the yield of fructan increased from 18.2% (80 ºC) to
294 20.5% (100 ºC), with S/M ratio of 30 mL/g, disparate in their results, maybe it was
295 because the variety, harvest time and storage temperature of material
296 (Saengthongpinit & Sajjaanantakul, 2005), the ratio of S/M, and the content of inulin.

297 **3.2 Optimization of extraction yield using RSM**

298 3.2.1 Model fitting

299 The variance analysis, including coefficient of variation, predicted residual, R-squared
300 (R^2), adjusted R-squared (Adj-R^2), were calculated to check the adequacy and
301 accuracy of the developed models (Jiao et al., 2017).

302 Applying multiple regression analysis on the experimental data, the response variable
303 and the test variable were fitting the following second-order polynomial equation:

304
$$Y = 19.2367 + 0.6975 * X_1 + 0.4756 * X_2 + 0.7981 * X_3 - 0.1 * X_1 * X_2 + 0.17 * X_1 * X_3 - 0.37375 * X_2 * X_3 - 0.4715 * X_1^2 - 0.2152 * X_2^2 - 0.2202 * X_3^2$$

305 (3)

306 The multivariate regression relationship between the dependent variable and the
307 independent variables was significant, declaring that the model was fitting well, could
308 represent the actual relationship between the experimental results and the theoretical
309 values, as the $R^2 = 0.95$, $R^2_{Adj} = 0.87$, $F = 11.0734$, $p = 0.0082$ ($p < 0.01$). Besides, the
310 probability of lack of fit was 0.4124 ($p > 0.05$), showed the model was less affected
311 by the other factors, and could simulate and predicate the change of extraction yield
312 with high degree of precision and credibility. In conclusion, this model equation could
313 explain most of the variability of the date based on these indexes.

314 **Table 2** Regression coefficients for three dependent variables.

Regression coefficients	p
X_1	0.00259^{**}
X_2	0.01277^{*}
X_3	0.00142^{**}
X_1*X_3	0.38220
X_1*X_2	0.59752

X_2*X_3	0.08913
X_1^2	0.05116
X_2^2	0.29664
X_3^2	0.28679

315 X_1=Extraction Temperature; X_2=S/M Ratio; X_3=Extraction Time.

316 *Significant at 0.05 level; **Significant at 0.01 level.

317 As showed in Table 2, the regression coefficient values of Eq. (3) were listed. X_1
318 (extraction temperature), X_2 (S/M ratio) and X_3 (extraction time) had significant
319 impact on the yield of fructan ($p < 0.05$), of which X_1, X_3 are highly significant ($p <$
320 0.01). It was clear that these three factors had an influence on the extraction yield; the
321 probability of quadratic term X_1^2 was 0.051, showed that extraction yield was mainly
322 affected by temperature, the same found Sun et al. (2010); the probability of
323 interaction term X_2*X_3 was 0.089, accounting for the interaction of S/M ratio and
324 extraction time had great influence on it; the other term coefficients were not
325 significant ($p > 0.05$).

326 3.2.2 Analysis of response and contour surface plots

327 The use of 3D-response surface and contour plots demonstrated the relationship
328 between extraction temperature, time and S/M ratio in brevity and clear way (Fig.2).
329 The content of fructan were greatly affected by these three factors, in good agreement
330 with the result of regression coefficient in Table 2, and the other studies in optimizing
331 the extraction of polysaccharides from *C. pilosula*. (Sun, Liu, & Kennedy, 2010; Yu et
332 al., 2015; Zou et al., 2011), that the yield of the polysaccharides could be increased
333 accompanied with the rise of temperature, S/M ratio and extraction time.

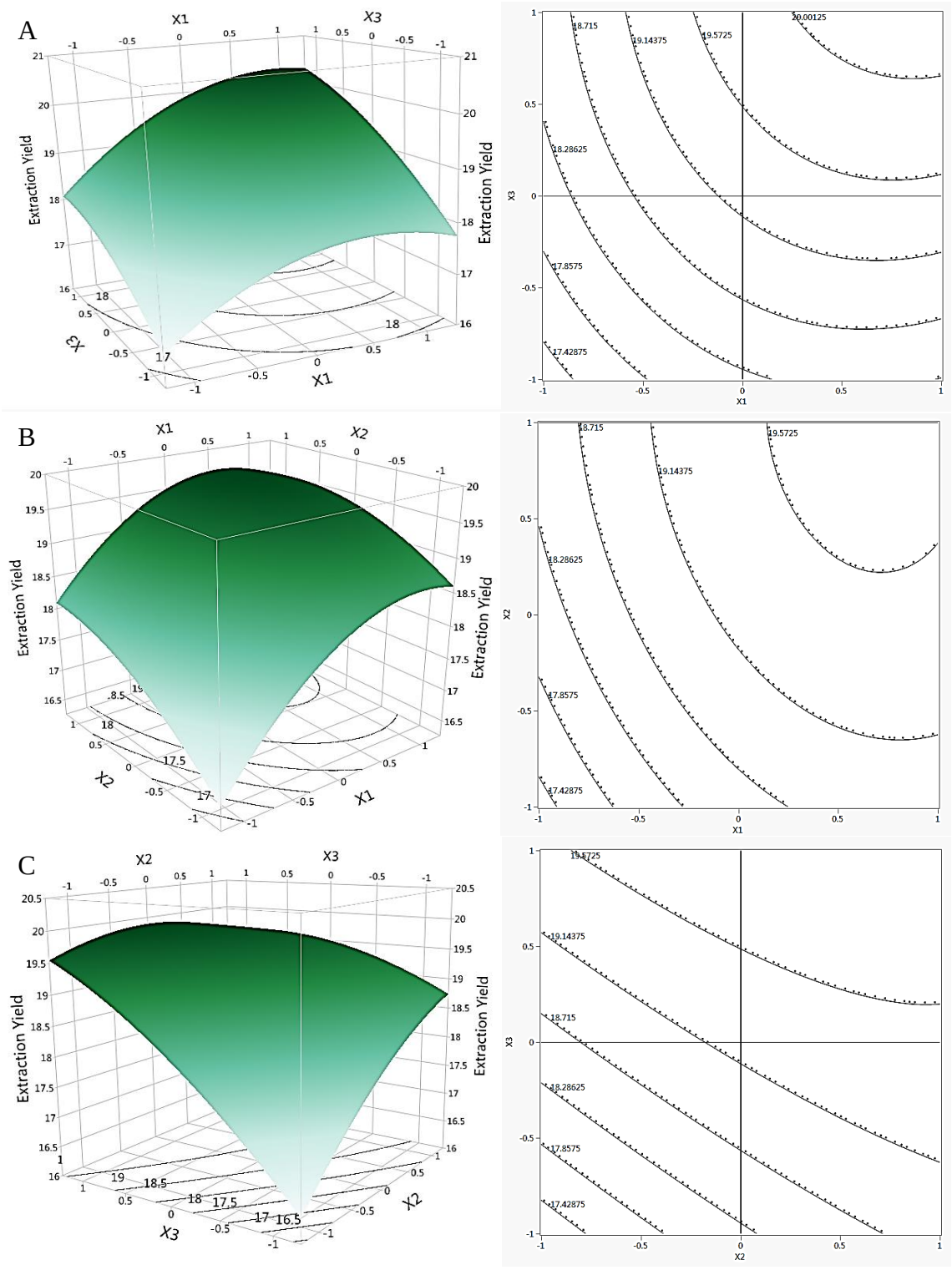

Fig.2. The interactive effects on the extraction yields of fructan from *C. pilosula* (left, response surface plots; right, contour plots). A, Effect of extraction temperature (X_1) and time (X_3) on the yield of fructan from *C. pilosula*; B, Effect of extraction temperature (X_1) and S/M ratio (X_2) on the yield of fructan from *C. pilosula*; C, Effect of S/M ratio (X_2) and extraction time (X_3) on the yield of fructan from *C. pilosula*.

Fig.2A displayed the effects of X_1 and X_3 on the yield of fructan at a fixed X_2 level 0 and the yield increased with rise of extraction temperature from 80 to 99.18 °C and time from 1.5 to 2.5 h, respectively. Fig.2B presented the quadratic effects of X_1 and X_2 on the fructan yield as X_3 was fixed at level 0. The yield enhanced with increase of extraction temperature from 80 to 99.18°C, and S/M ratio increasing from 30 to 40.02 mL/g. However, it decreased with further increase of extraction temperature. The reciprocal effects of X_2 and X_3 on the yield when X_1 was fixed at level 0 and was shown in Fig.2C. The result indicated that the fructan yield was maximum at the ratio of S/M and extraction time, were 40.02 mL/g and 2.5 h, respectively, and decreased when the ratio was increased beyond 40.02 mL/g. In general, it can be concluded that optimal extraction conditions of fructan from *C. pilosula* were extraction temperature 99.18°C, ratio of S/M 40.02 mL/g, and extraction time 2.5 h (Fig.2). In addition, the contour plot in Fig.2C showed that it was closer to the linear relationship between X_2 and X_3, as shown in Table 2 ($p=0.089$), indicating that the reciprocal action between extraction time and S/M ratio could be the main role, of which the extraction time was more influential ($p=0.00142$). The extraction time was the most significant factor to affect the extraction yield of fructan from *C. pilosula*, followed by extraction temperature and S/M ratio according to the regression coefficients in Table 2 and gradient of slope in the 3-D response surface plot (Fig. 2).

3.2.3 Optimization of extraction conditions

By analysing the plots, the optimal conditions for fructan extraction were: extraction temperature of 99.18 ℃ ($X_1=0.9175$), extraction time of 2.5 h ($X_3=1.00$), and S/M ratio of 40.02 mL/g ($X_2=0.0235$). Under the optimal conditions, the maximum predicted yield of polysaccharide is 20.2%.

3.2.4 Verification of the models

Three verification tests were carried out to notarize the adequacy of models for predicting the values of dependent variables. Under the optimum conditions, the predicted maximum yield of fructan was 20.2%. A mean value of 20.6 ± 0.2% (n=3), was obtained from real extraction conditions, which was at extraction temperature of 100 °C, S/M ratio of 40 mL/g and extraction time 2.5 h. The yield was close to the

predicted value since the R^2 value of this model was high (Table 2). In a word, the Eq. (3) could simulate the trend of fructan content, applying to the experiment requirement and actual production.

3.3 Chemical characterization of CPPF

The CPPF was purified by DEAE anion exchange chromatograph, with yield of 62% (m/m) of CPP (34 g), extracted from 100 g *C. pilosula* in optimum conditions modified in 3.2.4. It consisted in Glc and Fru, which were detected by GC after methanolysis and the Urea-HCl colorimetric method, respectively. The inter-glycosidic linkages identified by GC-MS after methylation analysis showed that the polymer was composed of mainly terminal-Fru*f*, terminal-Glc*p* and 2, 1-linked Fru*f*, with ratio of 0.8, 1 and 3.9, respectively, and the DP_{av} calculated was 6 based on the areas of the compounds.

The serial combination of multiple size-exclusion chromatography (SEC) columns allows broad molecular weight distribution, and provides higher resolution. Multi-angle laser-light scattering (MALLS) is a technique for determining, independently, the absolute molar mass and the average size of particles in solution, by detecting how they scatter light (Li, Wu, Lv, & Zhao, 2013). But the fact that the light scattering signal was too weak to detect the low molecular mass (especially in low concentration) of the fructan, so that the small molecular of CPPF were not taken into account in the average *Mw* and *Mn* values using the instrument software (Evans, Gallagher, Ratcliffe, & Williams, 2016). Combing these results above, it was probability that the CPPF contained higher amount of small molecular inulin. Thus, the DP of CPPF was around 17 (2810 Da, which was obtained according to the SEC-MALLS, being the maximum *Mw* of the chains present), with DP_{av} 6 approximately.

The NMR signals were characterized and compared with chemical shift values from literature (de Oliveira et al., 2011; Pontes et al., 2016; Yang, Hu, & Zhao, 2011; Ye et al., 2005). The ^{13}C NMR spectrum contained six signals, one major signal at 103.15 ppm, assigned to the anomeric carbon of Fru*f*; five minor signals at 60.82, 76.91, 74.21, 80.99 and 62.05 ppm, being the signals of C1-Fru*f*, C3-Fru*f*, C4-Fru*f*, C5-Fru*f*

and C6-Fru*f*, respectively (Fig.3A); and two methylene carbon atoms C1-Fru*f* and C6-Fru*f* that were confirmed by DEPT 135 spectrum (Fig.3B). The carbon atom signal at 92.39 ppm was assigned to Glc*p* because signals beyond 100 ppm pointed to ketose residues. The other signals between 60.42 ~ 71.10 ppm belonged to the C2~C6 of the Glc*p* (Fig.3A). The ^{1}H NMR spectrum of CPPF contained a main anomeric proton at 5.30 ppm, belonging to the H1-Glc*p*, terminal monosaccharide (Fig.3C). The other signals at δ 4.11 and 3.96, were assigned to the H3-Fru*f* and H4-Fru*f*, respectively, suggesting a DP of about 16-17 by the mean ratio between the integral proton signal (H3-Fru*f* and H4-Fru*f*) and the integral of Glc*p* signal (H^{1}-Glc*p*) (Caleffi et al., 2015), coinciding with *Mw* testing. The signals between δ 3.56~3.79 corresponded to H1-Fru*f*, H5-Fru*f* and H6-Fru*f* (Fig.3C). All these results indicated that the CPPF was linked on C-2 of Fru*f* and C-1 of the terminal-Glc*p*, with backbone of (2→1)-Fru*f*, identified as a typical inulin-type fructan: β- (2→1)-linked configuration at the anomeric carbon of fructosyl residues, connected with terminal Glc*p* residue by α-D-(1→2) bond, combing the results of monosaccharides compositions and glycosidic linkage.

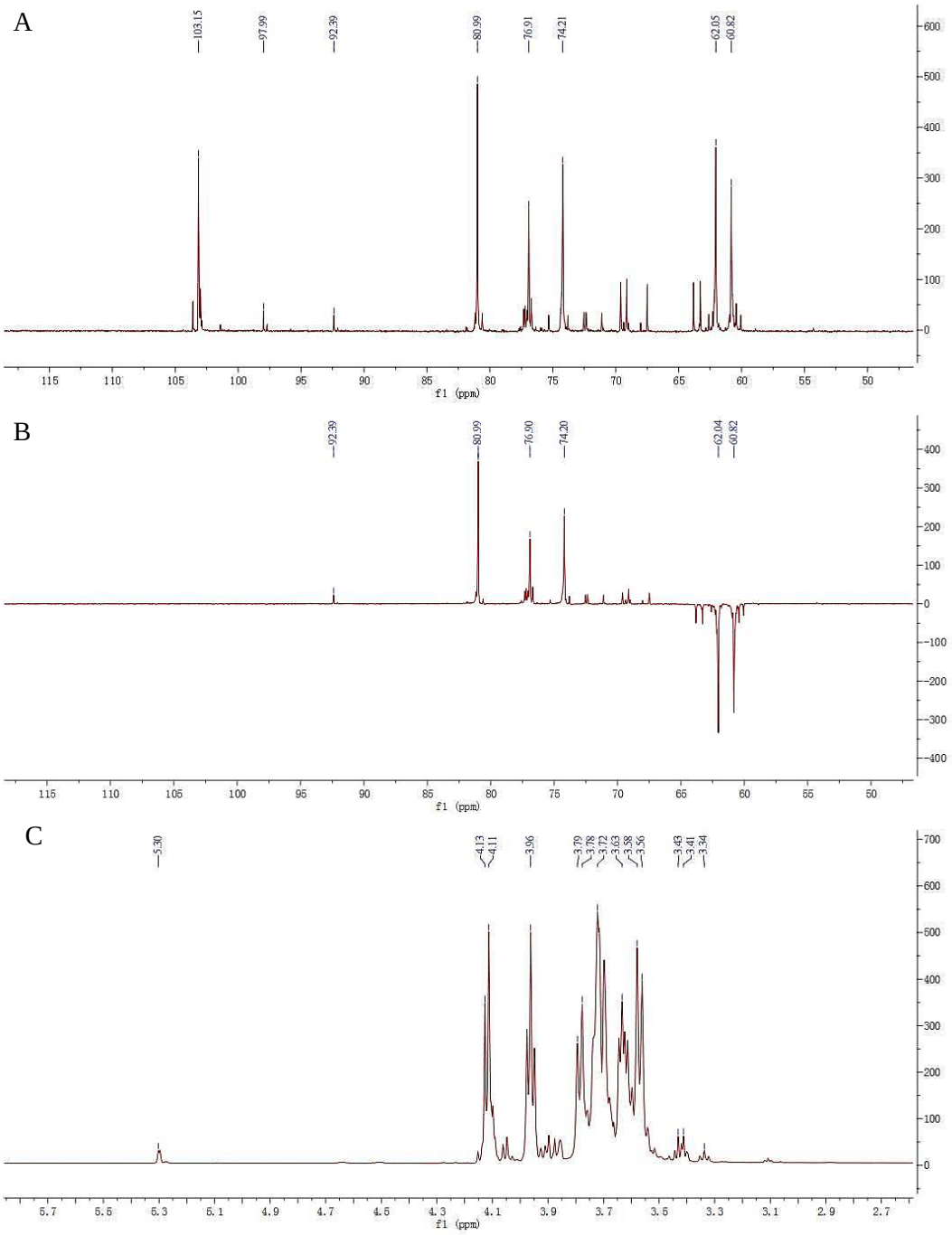

Fig.3. The^{13}C-NMR (A), DEPT (B) and ^{1}H-NMR (C) spectrum of CPPF.

3.4 Prebiotic effect

Inulin and fructo-oligosaccharides are defined as non-digestible carbohydrates which selectively stimulates the beneficial bacteria, like *Lactobacillus*, improving gut health (Lopes et al., 2017; Meyer et al., 2009). The CPPF (DP_n 2-17) was fermented by these six strains of lactobacilli, and the bacteria increased ($p<0.05$) in optical density of the medium compared to the basal medium (without sugar) (Table 3). The P95s (DP_n=2-9)

was the most suitable carbon source showing higher bacterial growth compared with CPPF (DP_n=2-17) and Orafti®HP (DP_{av}⩾23), while the bacteria in the medium containing Orafti®HP did not increase comparing to the basal medium ($p>0.05$) (Table 3). It was shown that the bacterial growth increased with decreasing of the DP, similar with previous reports (Caleffi et al., 2015; García Gamboa et al., 2018; Lopes et al., 2017; Li et al., 2015; Moreno-Vilet, 2014). In addition, the medium containing CPPF presented lower pH after 24 h incubation in anaerobic chamber compared to the basal medium, except Hjg8 (Table 4), which was consistent with the Δ_{600} in Table 4. Meanwhile, these six strains of lactobacilli in the basal medium containing glucose (1g/L) grew better than in MRS containing inulin, with lower final pH of the medium (not shown), because it is a monosaccharide and could be absorbed easily. The reduction of pH was due to the metabolites produced by lactobacilli, such as lactic acid and acetic acid (Satoh et al., 2013), and by the metabolite products from inulin, like SCFA (Karimi, Azizi, Ghasemlou, & Vaziri, 2015). The increase in bacterial density and lower pH indicated the growth of probiotics and an effective utilization of CPPF.

Strain specificity in fermentation capacity was observed and each strain utilized CPPF with varying intensity (Table 3), agree with these previous studies (Caleffi et al., 2015; Lopes et al., 2017; Li et al., 2015; Watson et al., 2013). In this study, CPPF could be utilized by all the six strains of lactobacilli. The strain *L.* BS15 was shown to utilize CPPF most efficiently, where the highest increase in ΔA_{600} (5.5-fold to basal medium) after incubating 24 h, so did the strain *L.* LGG (2.3-fold to basal medium). However, the strain *L.* Hjg8 demonstrated a small ability to ferment CPPF (0.32-fold to basal medium) (Table 3). The strain specificity of inulin utilization is due to the capacity of the microorganism to ferment complex sugar, which depends on their enzymatic equipment, especially the presence of hydrolases and transportases, like the enzyme responsible for the hydrolysis of fructan in position β- (2→1) (Lopes et al., 2017).

Table 3 Capacity to ferment CPPF or commercial prebiotics by *Lactobacillus*

Groups	The bacterial density (ΔA_{600}, n=3)			
	Basal medium	P95S	Orafti®HP	CPPF
L. buchneri BSS1	0.0496 ± 0.0020^{aA}	0.1671 ± 0.0062^{cA}	0.0534 ± 0.0027^{aA}	0.0926 ± 0.0018^{bA}
L. johnsonii BS15	0.0053 ± 0.0009^{aB}	0.0844 ± 0.0015^{cB}	0.0075 ± 0.0012^{aB}	0.0291 ± 0.0023^{bB}
L. johnsonii Hjg8	0.0524 ± 0.0012^{aC}	0.1875 ± 0.0091^{cC}	0.0537 ± 0.0026^{aA}	0.0690 ± 0.0022^{bC}
L. rhamnosus LGG	0.0333 ± 0.0087^{aD}	0.0973 ± 0.0011^{cD}	0.0302 ± 0.0008^{aC}	0.0758 ± 0.0067^{bC}
L. plantarum BSGP201683	0.0720 ± 0.0016^{aE}	0.2118 ± 0.0074^{cE}	0.0751 ± 0.0015^{aD}	0.1257 ± 0.0042^{bD}
L. plantarum BS10	0.0552 ± 0.0010^{aF}	0.3039 ± 0.0011^{cF}	0.0541 ± 0.0047^{aA}	0.0957 ± 0.00049^{bA}

Notes: Data are expressed as increase in A_{600} of the bacterial suspension with 24 h incubation; values are means from triplicate determination ± standard deviation.

^{abcd} Data in lines with different superscripts are significantly ($p < 0.05$)

^{ABCDEF} Data in columns with different superscripts are significantly ($p < 0.05$).

Table 4 The final pH of medium containing CPPF or commercially prebiotics by *Lactobacillus*.

Groups	pH (n=3)			
	Basal medium	P95s	Orafti®HP	CPPF
L. buchneri BSS1	6.58 ± 0.06^{aC}	5.92 ± 0.02^{cC}	6.48 ± 0.05^{abC}	6.43 ± 0.10^{bD}
L. johnsonii BS15	6.49 ± 0.34^{aC}	5.74 ± 0.13^{cB}	6.50 ± 0.05^{aC}	6.28 ± 0.06^{bC}
L. johnsonii Hjg8	5.81 ± 0.07^{aA}	5.45 ± 0.04^{bA}	5.83 ± 0.02^{aA}	5.79 ± 0.03^{aAB}
L. rhamnosus LGG	6.04 ± 0.01^{aB}	5.71 ± 0.01^{cB}	6.02 ± 0.03^{aB}	5.84 ± 0.05^{bB}
L. plantarum BSGP201683	5.82 ± 0.02^{aA}	5.40 ± 0.01^{cA}	5.83 ± 0.01^{aA}	5.73 ± 0.01^{bA}
L. plantarum BS10	5.80 ± 0.07^{aA}	5.40 ± 0.01^{cA}	5.82 ± 0.01^{aA}	5.72 ± 0.01^{bA}

Notes: Data are expressed as the final pH of the medium after 24 h incubation; values are means from triplicate determination ± standard deviation.

^{abc} Data in lines with different superscripts are significantly ($p < 0.05$)

^{ABCD} Data in columns with different superscripts are significantly ($p < 0.05$).

Other than the prebiotics activity, the inulin could stimulate the intestinal mucosa immunity directly, strengthen the epithelial barrier function, and could protect from the enterogenous endotoxin absorption and reducing inflammatory bowel disease (Franck & Bosscher, 2006; Masanetz, Preißinger, Meye, & Pfaffl, 2011; Vogt et al.,

2015). Nevertheless, the studies about prebiotic activity *in vitro* are not enough to claim the real mechanism. It must be considered to combine the impact of inulin on intestinal immunity, both *in vitro* and *in vivo*, and this would be done in further studies.

4 Conclusion

In this study, the optimal extraction condition of fructan from *C. pilosula* were obtained by RSM, and the maximum yield was 20.6% (m/m) employing the parameters of temperature 100 ºC, ratio of solvent to material 40 mL/g, extraction time 2.5 h. The fructan was consisted of β-D-Fru*f* and α-D-Glc*p*, with β-(2→1)-linked configuration and degree of polymerization of 2-17, identified as α-D-Glc*p*-(1→2)-[β-D-Fru*f*-(2→1)-β-D-Fru*f*]$_n$-(2→1)-β-D-Fru*f*, i.e. inulin-type fructan. In addition, it could be fermented by six strains of *Lactobacillus*, reduce the pH of the medium, and could be a potential prebiotic, depending on their structure and degree of polymerization. Nevertheless, we merely explored the preliminary prebiotic activity of CPPF based on the structure analysis. In the future, we will investigate the specific target on the bacteria and also how inulin is acting on the intestinal mucosa.

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