Carbohydrate Polymers 91 (2013) 175–182

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Pectic-oligosaccharides prepared by dynamic high-pressure microfluidization and their *in vitro* fermentation properties

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ARTICLE INFO

Article history:
Received 27 April 2012
Received in revised form 26 June 2012
Accepted 3 August 2012
Available online 14 August 2012

Keywords:
Pectin
Pectic-oligosaccharides (POS)
Dynamic high-pressure microfluidization (DHPM)
In vitro fermentation

ABSTRACT

Pectic-oligosaccharides (POSs) were prepared from apple pectin by dynamic high-pressure microfluidization (DHPM). Operating under selected conditions (pectin concentration 1.84%, solution temperature 63 °C, DHPM pressure 155 MPa and number of cycles 6 passes), 32.92% of the pectin was converted into POS. The resulting POS contains 29.56% galacturonic acid and 58.53% neutral sugars. The prebiotic properties of POS were then evaluated using a fecal batch culture fermentation. The POS increased the number of *Bifidobacteria* and *Lactobacilli*, and produced a higher concentration of acetic, lactic, and propionic acid than their parent pectin. Furthermore, POS decreased the number of *Bacteroides* and *Clostridia* while their parent pectin increased them. Moreover, the effects of POS on the growth of these bacteria and production of short-chain fatty acids are comparable to those of the most studied prebiotic, fructooligosaccharide. These results indicated that the POS prepared by DHPM has a potential to be an effective prebiotic.

1. Introduction

In the last decade, a number of novel dietary carbohydrates have been introduced as ingredients for food applications, responding to the growing awareness among consumers of the link between health, nutrition and diet. One import group is formed by the non-digestible oligosaccharides which may function as prebiotics. Several oligosaccharides with prebiotic properties, such as fructooligosaccharides, galactooligosaccharides, and fructans, are commercially available (Rastall & Hotchkiss, 2003), but there has been a considerable increase in the demand for production of "second generation" of novel prebiotic ingredients in recent years (Hernandez-Hernandez, Luz Sanz, Kolida, Rastall, & Javier Moreno, 2011).

It was reported that pectic-oligosaccharides (POSs) could be an excellent candidate for second-generation prebiotics (Hotchkiss, Olano-Martin, Grace, Gibson, & Rastall, 2003). Its parent material, pectin, is a family of complex and heterogeneous polysaccharides that widely present within the primary cell wall and intercellular regions of higher plants. Some studies have been carried out for preparing POS from kinds of source species, such as sugar beet pectin (Meyer et al., 2011), orange peel wastes (Martínez, Yáñez, Alonsó, & Parajó, 2010), and olive by-products (Lama-Muñoz, Rodríguez-Gutiérrez, Rubio-Senent, & Fernández-Bolaños, 2012) in the recent years.

Generally, POS was obtained according to three strategies: (1) extraction from plants (Ducasse, Williams, Meudec, Cheynier, & Doco, 2010); (2) synthesis (Nemati, Karapetyan, Nolting, Endress, & Vogel, 2008); (3) depolymerization of the polysaccharides. The depolymerization was regarded as the most competitive method because a wide variety of oligomers can be obtained from one polymer (Courtois, 2009). Basically, acid hydrolysis (Hu, Liu, Wang, & Ding, 2009), enzymatic hydrolysis (Zheng & Mort, 2008) and physical degradations are three frequently used depolymerization methods, where physical degradation was considered to be an efficient and environmentally friendly method. Physical methods such as γ-ray irradiation (Byun, Kang, Jo, Kwon, Son, & An, 2006) have been applied to prepare POS.

Dynamic high-pressure microfluidization (DHPM) is an emerging dynamic high-pressure homogenization technology, which generated powerful shear, turbulence, impaction, and cavitation forces simultaneously (Chen, Huang, Tsai, Tseng, & Hsu, 2011; Liu, Liu, Xie, Liu, Liu, & Wan, 2009). This technology has been proven to be a promising physical method to manipulate the molecular weight of polymers (Tsai, Tseng, & Chen, 2009). In our previous research, it was found that pectin being DHPM-treated induced serious degradation of high methoxyl pectin (Chen et al., 2012), indicating this method may be an alternative method to produce the POS, and need to be further developed.

The present study was conducted to optimize the production of POS by novel DHPM technology through a response surface methodology experiment. The prebiotic of the resulting POS was

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http://dx.doi.org/10.1016/j.carbpol.2012.08.021

Table 1
Four-factor, three-level Box–Behnken design used for RSM and experimental data of the investigated response.

Run order	Variables				Response
	X_1	X_2	X_3	X_4	Y
1	1.75 (0)	140.00 (0)	70.00 (+1)	6.00 (+1)	521.7
2	1.75 (0)	140.00 (0)	65.00 (0)	5.00 (0)	573.5
3	1.75 (0)	120.00 (−1)	65.00 (0)	6.00 (+1)	518.3
4	1.75 (0)	140.00 (0)	60.00 (−1)	4.00 (−1)	518.7
5	1.75 (0)	120.00 (−1)	60.00 (−1)	5.00 (0)	443.9
6	1.75 (0)	160.00 (+1)	65.00 (0)	6.00 (+1)	593.7
7	1.75 (0)	140.00 (0)	65.00 (0)	5.00 (0)	571.7
8	1.75 (0)	160.00 (+1)	70.00 (+1)	5.00 (0)	529.1
9	1.50 (−1)	140.00 (0)	65.00 (0)	6.00 (+1)	435.9
10	1.50 (−1)	120.00 (−1)	65.00 (0)	5.00 (0)	316.3
11	1.75 (0)	140.00 (0)	65.00 (0)	5.00 (0)	565.1
12	2.00 (+1)	140.00 (0)	70.00 (+1)	5.00 (0)	477.5
13	2.00 (+1)	140.00 (0)	60.00 (−1)	5.00 (0)	535.3
14	2.00 (+1)	120.00 (−1)	65.00 (0)	5.00 (0)	410.7
15	1.50 (−1)	160.00 (+1)	65.00 (0)	5.00 (0)	425.5
16	2.00 (+1)	160.00 (+1)	65.00 (0)	5.00 (0)	543.7
17	2.00 (+1)	140.00 (0)	65.00 (0)	6.00 (+1)	539.1
18	1.75 (0)	140.00 (0)	70.00 (+1)	4.00 (−1)	506.9
19	1.50 (−1)	140.00 (0)	60.00 (−1)	5.00 (0)	353.3
20	1.75 (0)	140.00 (0)	65.00 (0)	5.00 (0)	597.5
21	1.50 (−1)	140.00 (0)	65.00 (0)	4.00 (−1)	429.7
22	1.75 (0)	120.00 (−1)	65.00 (0)	4.00 (−1)	501.7
23	1.50 (−1)	140.00 (0)	70.00 (+1)	5.00 (0)	389.1
24	2.00 (+1)	140.00 (0)	65.00 (0)	4.00 (−1)	542.9
25	1.75 (0)	160.00 (+1)	65.00 (0)	4.00 (−1)	581.7
26	1.75 (0)	160.00 (+1)	60.00 (−1)	5.00 (0)	578.1
27	1.75 (0)	120.00 (−1)	70.00 (+1)	5.00 (0)	469.3
28	1.75 (0)	140.00 (0)	65.00 (0)	5.00 (0)	567.5
29	1.75 (0)	140.00 (0)	60.00 (−1)	6.00 (+1)	551.7

evaluated through enumerating of selected human gut microflora including *Bifidobacteria*, *Lactobacilli*, *Bacteroides*, *Clostridia* and *Eubacteria*, and analysing the concentration of short-chain fatty acid in a fecal batch culture fermentation test. Fructooligosaccharides (FOS), the most extensively studied prebiotics and the current market leader, were chosen as a positive control.

2. Materials and methods

2.1. Materials

Apple pectin (8471, Sigma–Aldrich, Shanghai, China) were dispersed in phosphate buffer and treated enzymatically for starch degradation, using α-amylase (A6211 from *Aspergillus oryzae*, Sigma–Aldrich, Shanghai, China) and amyloglucosidase (10115 from *Aspergillus niger*, Sigma–Aldrich, Shanghai, China) according to methods used by Urias-Orona, Rascón-Chu, Lizardi-Mendoza, Carvajal-Millán, Gardea, and Ramírez-Wong (2010). Then the digestion products were removed by a 48 h dialysis (molecular weight cut-off 6000–8000) with continuous change of distilled water. After dialysis, the retentates were freeze-dried. The galacturonic acid content of purified pectin was 71.68% determined by the m-hydroxybiphenyl method (Blumenkrantz & Asboe-Hansen, 1973), and the degree of methoxylation (DM) was 70.76% determined by a titrimetric method (FCC, 1981). All of the other chemicals were of analytical reagent grade.

2.2. Preparation of POS by DHPM

2.2.1. DHPM treatment

Pectic-oligosaccharides were prepared by dynamic high-pressure microfluidization according to our previous method (Chen et al., 2012). Purified pectin was dispersed in deionized water and stirred gently at room temperature to achieve complete solubilization. Then the solution was adjusted to pH 1.0 using 0.5 M H_2SO_4 and placed in a water bath to achieve the required temperature. The solution was then treated in an M-100EH-30 microfluidizer (Microfluidics Co., Newton, USA). After DHPM, the reaction mixture was neutralized with calcium carbonate to precipitate the higher molecular weight species that are not soluble in neutral solution, and the resulting precipitate was removed by centrifugation (Du, Song, Hu, Liao, Ni, & Li, 2011). The supernatant was circulated through a concurrent ultrafiltration with a cut-off size of 5000 Da (Pall Gelman, Ann Arbor, MI) to remove the species that their molecular weight were larger than 5000 Da, and permeates were collected, freeze-dried and weighted.

2.2.2. Experimental design

The response surface methodology (RSM) was adopted to study the effect of pectin concentration (X_1), DHPM pressure (X_2), solution temperature (X_3) and number of cycles (X_4) on the mass of POS (Y). A three level four factor Box–Behnken design (BBD) was used in this study. A total of 29 experiments, consisting of 5 replicates with combinations of different levels of each independent variable, were generated using the software Design Expert version 8.0.5 (Stat-Ease, Inc., Minneapolis, USA) (Table 1). The range for pectin concentration, DHPM pressure, solution temperature and number of cycles was set at 1.5–2.0%, 120–160 MPa, 60–70 °C, and 4–6 passes, respectively, based on our preliminary experiments. Individual experiments were carried out in randomized order to minimize the effect of unexplained variability in the experimental responses due to extraneous factors.

Experimental data were fitted to a quadratic polynomial model. The general form of the quadratic polynomial model was as follows:

$$\Upsilon = \beta_0 + \sum_{i=1}^{4}\beta_i X_i + \sum_{i=1}^{4}\beta_{ii}X_i^2 + \sum_{i=1}^{3}\sum_{j=i+1}^{4}\beta_{ij}X_iX_j \quad (1)$$

where Y is the predicted response; β_0, β_i, β_{ii} and β_{ij} are the regression coefficients for intercept, linearity, square and interaction, respectively; X_i and X_j are the independent variables.

2.3. Determination of sugar composition

The galacturonic acid content of oligosaccharides was determined by the m-hydroxybiphenyl method (Blumenkrantz & Asboe-Hansen, 1973). Neutral sugars of pectin and oligosaccharides were analyzed by gas chromatography (GC) according to Liang et al. (2012) with some modifications. Sample (12 mg) was hydrolyzed with 2 M trifluoroacetic acid (5 ml) for 12 h at 110 °C in a sealed tube. After the trifluoroacetic acid was blown off with nitrogen (to dryness), the sample was dissolved in 0.5 ml of 0.02% (w/v) pyridine–$NH_2OH{\cdot}HCl$ solution in a sealed test tube at 90 °C for 30 min. The solution was cooled to the room temperature and acetylated with 0.5 ml acetic anhydride in a sealed test tube at 90 °C for 30 min. 1 μl of the sample was analyzed in an Agilent 6890 system GC (Agilent Technologies, Palo Alto, CA, USA) fitted with a DB-1701 column (30 m, 0.25 mm I.D., 0.25 μm film thickness; kept at 170 °C for 2 min, 170–250 °C at 10 °C/min, then held at 250 °C for 30 min) with a flame ionization detector (FID).

2.4. Fecal batch culture fermentation

Fecal batch culture fermentation was performed according to the methods used by Mandalari et al. (2007) with minor modifications. Fermenter was filled with 40 ml of a pre-reduced culture medium containing peptone, 2.0 g l^{-1}; yeast extract, 2.0 g l^{-1}; NaCl, 0.1 g l^{-1}; K_2HPO_4, 0.04 g l^{-1}; KH_2PO_4, 0.04 g l^{-1}; $MgSO_4{\cdot}7H_2O$, 0.01 g l^{-1}; $CaCl_2{\cdot}2H_2O$, 0.01 g l^{-1}; $NaHCO_3$, 2.0 g l^{-1}; cysteine HCl, 0.5 g l^{-1}; bile salts, 0.5 g l^{-1}; Tween 80, 2 ml l^{-1}; vitamin K_1, 0.01 ml l^{-1}; haemin solution, 1 ml l^{-1}. A 10% (w/v) fecal slurry was prepared by homogenizing fresh human feces from three healthy donors in phosphate-buffered saline, pH 7.0, with a homogenizer in an anaerobic cabinet. The healthy donors (average age 23 ± 1 years; one female, two male) who had not taken antibiotics within three month before the study. After the addition of fecal solution, 5 ml sample (Pectin, POS or FOS) which was previously mixed in autoclaved medium and sterilized by filtration through 0.22 μm filters, was added to a final concentration of 1%, w/v. Fructo-oligosaccharides (F8052, Sigma–Aldrich, Shanghai, China), the most extensively studied prebiotics, were chosen as a positive control. No carbohydrate was added as a negative control. Anaerobic conditions were maintained by sparging the vessels with oxygen-free nitrogen gas at 15 ml/min. Each fermentation experiment was carried out in triplicate and incubated at 37 °C. Fermentation samples were taken at 0, 6, 12 and 24 h, and analyzed for bacterial populations and concentration of short-chain fatty acids (SCFA).

2.4.1. Enumeration of bacteria

The fermentation samples were serially diluted. Appropriate dilutions were spread onto sterile Petri dishes, which were immediately poured with the following selective media: Beeren's agar, Rogosa agar, reinforced clostridia agar, and Brucella agar for *Bifidobacteria*, *Lactobacilli*, *Clostridia* and *Bacteroides* respectively (Olano-Martin, Mountzouris, Gibson, & Rastall, 2000), and eubacteria selective agar for *Eubacteria* (Shinohara, Ohashi, Kawasumi, Terada, & Fujisawa, 2010). The Petri dishes were then placed into anaerobic jars, and incubated at 37 °C for 24 h. The colonies arising on plates were counted to determine bacterial counts.

2.4.2. Analysis of SCFA

Analysis of SCFA was carried out according to the method used by Rehman, Hellweg, Taras, and Zentek (2008) with minor modifications. The fermentation samples was centrifuged at 13,000 rpm for 15 min and 1.0 μl of a supernatant was injected into an Agilent Technologies model 6890 gas chromatograph after filtered through 0.22 μm filters. An HP-INNOwax column (30 m, 0.25 mm I.D., 0.25 μm, Agilent Technologies) was used. The split injection was 40:1. Initial column temperature (120 °C) was hold for 1 min, thereafter increasing in stages of 10 °C per min until reaching 265 °C, which was maintained for 2 min. Flow rates of H_2, air, and N_2 was set at 40 ml min^{-1}, 450 ml min^{-1}, and 45 ml min^{-1}, respectively. Quantification of the short-chain fatty acids in the samples was carried out by using external calibration curves of acetic, propionic, butyric, and lactic acids.

2.5. Statistical analysis

All of the experiments were done in triplicate. Statistical analysis was carried out using SPSS (version 16.0, Chicago, United States). The results were expressed as mean ± standard deviations and compared using the Tukey test at 5% confidence level. RSM was performed using the Design Expert software (Version 8.0.5, Stat-Ease, Inc., Minneapolis, MN).

3. Results and discussions

3.1. Production of POS by DHPM

3.1.1. Model fitting and analysis of variance

The design matrix and the corresponding results of RSM experiments to determine the effects of the four independent variables, including pectin concentration (X_1), DHPM pressure (X_2), solution temperature (X_3) and number of cycles (X_4), were shown in Table 1. Through multiple regression analysis on the experimental data, the model for the predicted response Y could be expressed by the following quadratic polynomial equation (in the form of coded values):

$$Y = 575.06 + 58.28X_1 + 49.30X_2 - 7.28X_3 + 6.57X_4 + 5.95X_1X_2 - 23.40X_1X_3 - 2.50X_1X_4 - 18.60X_2X_3 - 1.15X_2X_4 - 4.55X_3X_4 - 100.73X_1^{2-}36.60X_2^{2-}41.28X_3^2 + 4.65X_4^2 \quad (2)$$

where Y is the mass of POS, X_1, X_2, X_3 and X_4 are the coded variables.

Statistical testing of the model was performed in the form of analysis of variance (ANOVA). The ANOVA for the fitted quadratic polynomial model of production of POS is shown in Table 2. The quadratic regression model showed the value of the determination coefficient (R^2) was 0.9697, which implied that 96.97% of the variations could be explained by the fitted model. The closer the value of R^2 to the unity, the better the empirical model fits the actual data (Lee, Yusof, Hamid, & Baharin, 2006). For a good statistical model, R^2_{adj} should be close to R^2. As shown in Table 2, R^2_{adj} was 0.9395, which implied that only less than 5.0% of the total variations were not explained by the model, and a high degree of correlation between the observed and predicted values. In addition, a relatively low value of C.V. (coefficient of variation) (3.65%) indicated a better reliability of the experimental values. According to Atkinson and Donev (1992), the corresponding variables would be more significant if the F-value becomes greater and the p-value becomes smaller, and values of p-value less than 0.05 showed model terms were significant. So the F-value ($F = 32.04$) and p-value ($p < 0.0001$) implied this model was significant. Significance of the model was also judged by lack-of-fit test. As shown in Table 2, F-value and p-value of the lack of fit was 2.41 and 0.2062, respectively, which implied that it was not significant and a 20.62% chance could occur due to noise. The significance of each coefficient was also determined using F-value and p-value. The results were given in

Table 2
Results of the ANOVA to the response surface quadratic polynomial model.

Source	D.F.[a]	Sum of squares	*F*-value	*p*-Value
Model	14	1.51E+005	32.04	<0.0001
X_1	1	40763.36	120.78	<0.0001
X_2	1	29165.88	86.42	<0.0001
X_3	1	636.56	1.89	0.1912
X_4	1	517.45	1.53	0.2360
X_1X_2	1	141.61	0.42	0.5276
X_1X_3	1	2190.24	6.49	0.0232
X_1X_4	1	25.00	0.074	0.7895
X_2X_3	1	1383.84	4.10	0.0624
X_2X_4	1	5.29	0.016	0.9021
X_3X_4	1	82.81	0.25	0.6280
X_1^2	1	65815.35	195.01	<0.0001
X_2^2	1	8691.41	25.75	0.0002
X_3^2	1	11053.22	32.75	<0.0001
X_4^2	1	139.95	0.41	0.5300
Residual	14	4725.02		
Lack of fit	10	4051.39	2.41	0.2062
Pure error	4	673.63		
$R^2 = 0.9697$, $R^2_{adj} = 0.9395$, C.V.[b] = 3.65%				

[a] D.F., degree of freedom.
[b] C.V., coefficient of variation.

Table 2. It could be seen from this table that two linear coefficients (X_1, X_2), one cross product coefficients (X_1X_3) and three quadratic terms (X_1^2, X_2^2 and X_3^2) were significant, with very small *p*-values ($p < 0.05$). The other term coefficients were not significant ($p > 0.05$). Therefore, X_1, X_2, X_1X_3, X_1^2, X_2^2 and X_3^2 were important factors in the production of POS by DHPM.

3.1.2. Optimization of the procedure

The relationship between the variables and the response can be better understood by examining the three-dimensional response surface plots, as shown in Fig. 1A–F, whose regression coefficients are generated from the predicted models as shown in Table 2. In the plots, two continuous variables were developed for mass of POS, while the other two variables were held constant at their respective zero level. As shown in Fig. 1A–C, when other variables were fixed at zero level, pectin concentration (X_1) demonstrated quadratic effects on the mass of POS. The mass of POS increased at first and then decreased with the increase of pectin concentration (X_1). The elliptical contour plot shown in Fig. 1B indicated the mutual interactions between pectin concentration (X_1) and solution temperature (X_3) were significant. The results of Fig. 1E and F showed that when the number of cycles (X_4) was changed, the change of response was comparatively small, which is in accordance with the results of Table 2 that X_4, X_1X_4, X_2X_4, X_3X_4 and X_4^2 were not significantly affect the response.

The optimal parameters for the highest mass of POS were predicted to be pectin concentration 1.84%, DHPM pressure 155.89 MPa, solution temperature 62.90 °C and number of cycles 6.00 passes. Under the conditions, the mass of POS was 617.6 mg/100 ml. However, considering the operability in actual production, pectin concentration 1.84%, DHPM pressure 155 MPa, solution temperature of 63 °C and number of cycles 6.00 passes were used and three new experiments were performed. The experimental (605.7 ± 13.5 mg/100 ml) and predicted values were found to be not statistically different at 5% level of significance, indicating that the model was adequate. Then the corresponding yield of oligosaccharides from apple pectin was 32.92 ± 0.73%. There are some literatures about manufacturing pectic-oligosaccharides from other raw materials. For example, Martínez, Gullón, Yáñez, Alonsó, & Parajó (2009) produced 26.70% oligosaccharides from sugar beet pulp by direct enzymatic treatment, who also prepared 25.10% pectic-oligosaccharides from orange peel wastes by nonisothermal processing with hot compressed water (Martínez et al., 2010).

3.2. Sugar composition of pectin and POS

Commercial apple pectin used in this study contained 71.68% galacturonic acid and its total neutral sugars content was 21.01%. Galactose (7.45%) and arabinose (6.61%) were the major components of the neutral sugars, followed by rhamnose (3.48%), xylose (2.91%), and glucose (0.56%). In addition, no mannose was found in the pectin (Table 3).

For the POS that derived from this commercial pectin by DHPM, it had the same sugar type as that of pectin, but with higher amounts of total neutral sugars (58.53%) and lower content of galacturonic acid (29.56%) (Table 3). Combined the yield of POS and sugar compositions of apple pectin, it was found that 91.71% of neutral sugars and 13.58% of galacturonic acid that presented in the pectin were converted into POS. It seems to be that glycosidic bonds between neutral sugars were more susceptible to breakdown than that between galacturonic acid during the process of DHPM. Thibault, Renard, Axelos, Roger, & Créeau (1993) reported that acid hydrolysis released different sugar residues present in pectic polysaccharides at very different rates, with the galacturonic acid being the most resistant. In our previous study, we proved that acid hydrolysis played an important role in the degradation of pectin during DHPM (Chen et al., 2012), then pattern of scission in this process may be similar to that in the acid hydrolysis, leading to neutral sugars have higher conversion rates than that of galacturonic acid.

3.3. In vitro fermentation characteristics of POS

3.3.1. Changes in bacterial population

Although it is very difficult to fully characterize all changes occurring in the colonic microflora, monitoring of populations of selected species could be an indicator of the state of colon health (Cardelle-Cobas et al., 2012). Table 4 shows changes in bacterial populations of *Bifidobacteria*, *Lactobacilli*, *Bacteroides*, *Clostridia*, and *Eubacteria* following 24 h fermentation of pectin, POS or FOS. For the *Bifidobacteria*, the number of which was increased at a higher number by POS than that by parent pectin. For instance, after 24 h incubation, the number of *Bifidobacteria* increased from 7.93 log to 8.65 log and to 8.37 log by POS and pectin, respectively. In addition, POS also showed a higher rate for *Bifidobacteria* increase. For example, pectin exhibited a significant different number of *Bifidobacteria* only after 24 h incubation, while POS significantly increased *Bifidobacteria* population after 12 h. Moreover, the number of *Bifidobacteria* at 6 h for POS (8.22 log) was equal to that of pectin at 12 h (8.22 log), and 8.58 log at 12 h for POS was higher than 8.37 log for pectin at 24 h. When POS was compared with FOS, the number of *Bifidobacteria* increased faster in the initial 6 h incubation of FOS. However, there is no significant difference between POS and FOS at 12 h or 24 h incubation. In the case of *Lactobacilli*, the number did not significantly change during 24 h fermentation of pectin, but increased significantly from 7.26 log to 7.58 log after fermentation with POS for 12 h. At that time point, the number of *Lactobacilli* in POS was also equivalent to that (7.66 log) in FOS.

On the other hand, POS decreased the number of *Clostridia* by 47.52% and the number of *Bacteroides* by 55.33% after 24 h fermentation. At the same time, a rise of *Eubacteria* population was observed, however, this increase was not significant. For FOS, the number of *Clostridia* also decreased from 7.49 log to 7.17 log after 24 h fermentation, while no significant changes was found in the population of *Bacteroides* and *Eubacterium* during the entire fermentation. Since *Bacteroides* and *Clostridia* are believed to be pathogenic microorganisms, the inhibition or failing sustention of

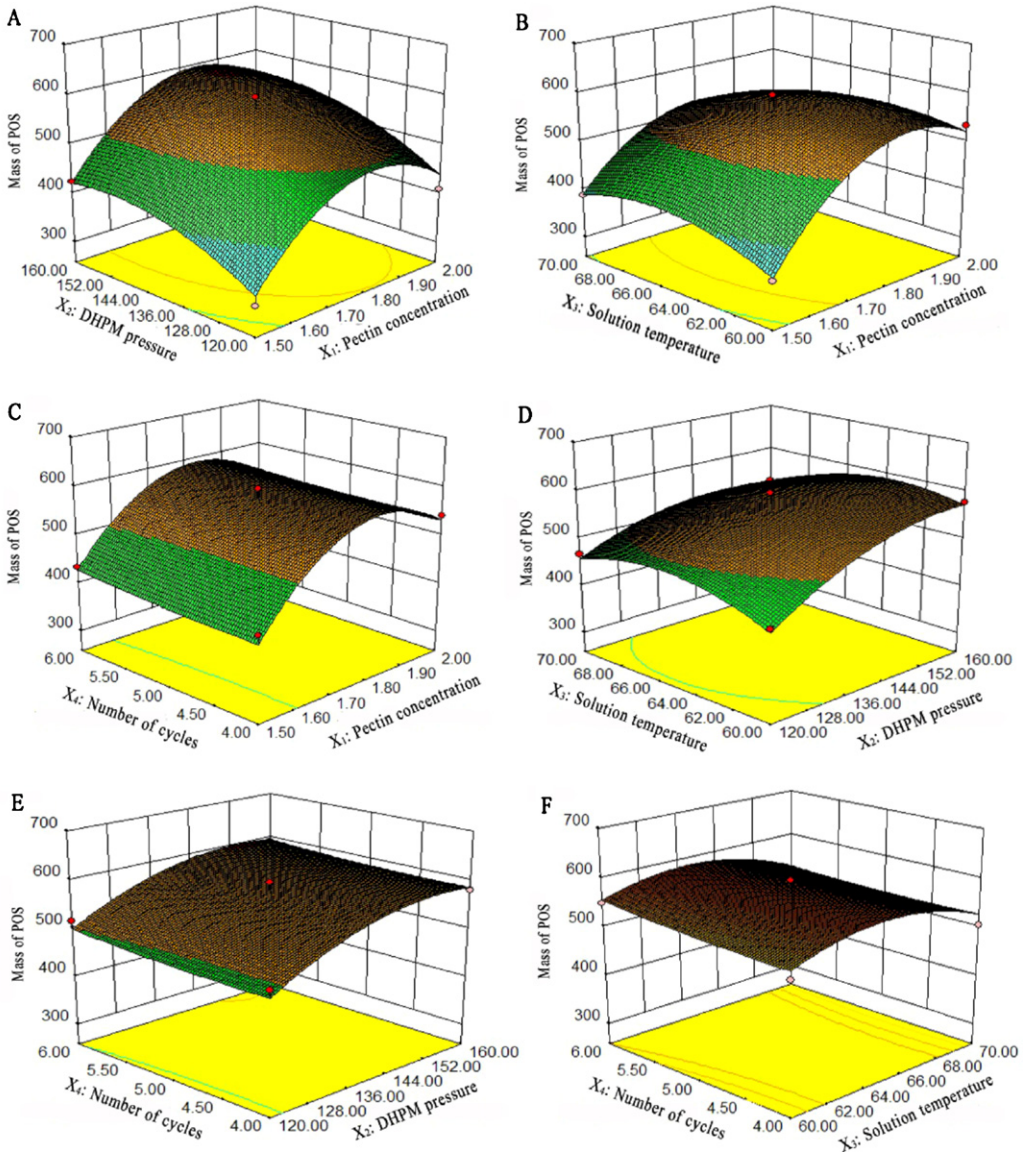

Fig. 1. Response surface plots for the effect of (A) pectin concentration and DHPM pressure, (B) pectin concentration and solution temperature, (C) pectin concentration and number of cycles, (D) DHPM pressure and solution temperature, (E) DHPM pressure and number of cycles, (F) solution temperature and number of cycles on the mass of POS.

which by POS will further favor the health of host. For the parent pectin, it was found that *Bacteriodes*, *Clostridia*, and *Eubacteria* could all grow well on it, where a significant increase was found after 12 h fermentation. The utilization of pectin by these three bacteria may be due to the presence of pectate lyases in these species. Dongowski, Lorenz, and Anger (2000) described a *Bacteriodes* specie as pectin degrading microbe in the human intestinal flora. Furthermore, pure strains of human gut bacteria (*Bacteroides* and *Eubacterium* species) were able to degrade pectin (Hill, 1995). Likewise, *Clostridium butyricum–C. beijerinckii* isolated from human faeces also splits pectin (Nakajima, Ishihara, Tanabe, Matsubara, & Matsuura, 1999).

3.3.2. Changes in short-chain fatty acid (SCFA) production

Products formed during fermentation from polysaccharides and oligosaccharides have been the subject of many studies (Cardelle-Cobas et al., 2012; Hernandez-Hernandez et al., 2011). Changes in SCFA concentrations after 0, 6, 12, and 24 h fermentation of pectin, POS or FOS are shown in Table 5. Generally, the total SCFA concentrations increased with incubation time for all carbohydrates, and

Table 3
Sugar compositions of apple pectin and pectic-oligosaccharides (POS).[a]

Sample	GalpA[b] (wt%)	Neutral sugars (wt%)					
		Arabinose	Galactose	Rhamnose	Glucose	Xylose	Mannose
Pectin	71.68 ± 1.53	6.61 ± 0.30	7.45 ± 0.21	3.48 ± 0.13	0.56 ± 0.31	2.91 ± 0.15	ND[c]
POS	29.56 ± 0.32	18.35 ± 0.65	21.12 ± 1.28	9.12 ± 0.33	1.52 ± 0.26	8.42 ± 0.37	ND

[a] Values are means ± standard deviations of triplicate measurements.
[b] GalpA is galacturonic acid.
[c] ND indicates not determined.

Table 4
Bacterial populations at 0, 6, 12 and 24 h of *in vitro* fermentation of pectin, pectic oligosaccharides (POS), or fructooligosaccharides (FOS) using human fecal microflora.[a]

	h	Negative control	Pectin	POS	FOS
Bifidobacteria	0	7.93 ± 0.21 aα*	7.93 ± 0.21 aα	7.93 ± 0.21 aα	7.93 ± 0.21 aα
	6	8.11 ± 0.15 aα	8.15 ± 0.09 aαβ	8.22 ± 0.16 aαβ	8.70 ± 0.22 bβ
	12	7.91 ± 0.22 aα	8.22 ± 0.11 abαβ	8.58 ± 0.28 bβ	8.67 ± 0.25 bβ
	24	7.99 ± 0.14 aα	8.37 ± 0.14 abβ	8.65 ± 0.24 bβ	8.73 ± 0.10 bβ
Lactobacilli	0	7.26 ± 0.17 aα	7.26 ± 0.17 aα	7.26 ± 0.17 aα	7.26 ± 0.17 aα
	6	7.21 ± 0.25 aα	7.23 ± 0.07 aα	7.49 ± 0.09 abαβ	7.62 ± 0.08 bβ
	12	7.23 ± 0.16 aα	7.26 ± 0.15 abα	7.58 ± 0.09 abβ	7.66 ± 0.13 bβ
	24	6.98 ± 0.12 aα	7.25 ± 0.11bα	7.42 ± 0.07 bαβ	7.70 ± 0.10 cβ
Bacteroides	0	8.20 ± 0.15 aα	8.20 ± 0.15 aα	8.20 ± 0.15 aα	8.20 ± 0.15 aα
	6	8.24 ± 0.07 aα	8.40 ± 0.07 aαβ	8.19 ± 0.14 aα	8.19 ± 0.22 aα
	12	8.19 ± 0.16 aα	8.53 ± 0.09 bβ	7.84 ± 0.11 cβ	8.25 ± 0.14 cα
	24	8.30 ± 0.12 acα	8.54 ± 0.13 aβ	7.85 ± 0.09 bβ	8.23 ± 0.09 cα
Clostridia	0	7.49 ± 0.12 aα	7.49 ± 0.12 aα	7.49 ± 0.12 aα	7.49 ± 0.12 aα
	6	7.31 ± 0.15 aα	7.82 ± 0.15 bαβ	7.41 ± 0.06 aαβ	7.41 ± 0.07 aαβ
	12	7.61 ± 0.23 abα	7.88 ± 0.07 bβ	7.27 ± 0.12 aαβ	7.32 ± 0.11 aαβ
	24	7.57 ± 0.24 abα	7.91 ± 0.18 bβ	7.21 ± 0.09 aβ	7.17 ± 0.11 aβ
Eubacteria	0	8.24 ± 0.14 aα	8.24 ± 0.14 aα	8.24 ± 0.14 aα	8.24 ± 0.14 aα
	6	8.23 ± 0.09 aα	8.57 ± 0.15 aβ	8.52 ± 0.18 aα	8.51 ± 0.12 aα
	12	8.25 ± 0.12 aα	8.61 ± 0.15 bβ	8.56 ± 0.14 abα	8.56 ± 0.11 abα
	24	8.20 ± 0.12 aα	8.66 ± 0.16 bβ	8.53 ± 0.12 abα	8.63 ± 0.23 bα

[a] Numbers are mean log 10 cell ml^{-1} sample ± standard deviations of triplicate fermentations.
* Different letters (a, b, c, . . .) in the same raw indicate significant differences between different carbohydrates (Tukey test, $p < 0.05$). Different Greek letters (α, β, γ, . . .) for the same bacteria and same column indicate statistical different between different time point (Tukey test, $p < 0.05$).

the POS produced the highest total concentration (59.85 mM) after 24 h fermentation. It was found that both the rate and the total SCFA concentration of pectin were lower than those of POS. For instance, pectin produced 9.01, 10.72 and 20.11 mM less total SCFA than that of POS at 6, 12, and 24 h, respectively. This phenomenon may be due to the complex structure of pectin which limits the accessibility of bacteria and hydrolytic enzymes to the pectin (Lebet, Arrigoni, & Amadŋ, 1998). Although FOS had a higher rate of SCFA production from 0 to 6 h, POS had a higher rate than that of FOS during the interval from 6 to 24 h, and there is no significant difference for total SCFA between POS and FOS after 12 and 24 h fermentation.

Acetic acid was the dominant SCFA produced in all fermenters. Its concentration increased with incubation time, and values after 6, 12, and 24 h incubation differed significantly ($p < 0.05$) from that at the start for all the carbohydrates used. POS produced more acetic acid than their parent pectin at all time point, with 4.40, 3.38 and 7.72 mM more at 6, 12 and 24 h, respectively. In addition, there is no significant difference for acetic acid concentration between FOS and POS at 24 h. In the case of lactic acid, the concentration in pectin sample was always lower than that of POS. For instance, the concentration of lactic acid for POS at 6 h (3.65 mM) was higher than that of pectin at 24 h (2.70 mM), and POS exhibited the highest lactic acid concentration (12.57 mM) at 24 h. Acetic and lactic acids are considered typical fermentation products of the bifidus pathway (Sanz et al., 2005). The pectin that presented the lowest numbers of *Bifidobacteria* and *Lactobacilli* also produced the lowest concentration of these acids. In addition, it was reported that lactic acid tends to be used quickly by the fecal microbiota (Kedia,

Table 5
Acetic acid, lactic acid, propionic acid, butyric acid, and total short-chain fatty acid (SCFA) at 0, 6, 12 and 24 h of *in vitro* fermentation of pectin, pectic oligosaccharides (POS), or fructooligosaccharides (FOS) using human fecal microflora.[a]

Sample	h	Negative Control	Pectin	POS	FOS
Total SCFA[b]	0	1.56 ± 0.35 aα*	1.56 ± 0.35 aα	1.56 ± 0.35 aα	1.56 ± 0.35 aα
	6	12.11 ± 0.24 aβ	14.61 ± 1.28 aβ	23.62 ± 1.25 bβ	28.55 ± 2.82 cβ
	12	19.69 ± 1.72 aγ	25.72 ± 1.10 bγ	36.44 ± 0.37 cγ	54.85 ± 0.83 dγ
	24	20.13 ± 1.20 aγ	39.74 ± 3.65 bδ	59.85 ± 0.49 cδ	56.89 ± 3.32 cγ
Acetic acid	0	1.26 ± 0.41 aα	1.26 ± 0.41 aα	1.26 ± 0.41 aα	1.26 ± 0.41 aα
	6	8.15 ± 0.43 aβ	10.29 ± 1.15 aβ	14.69 ± 1.95 bβ	19.93 ± 1.67 cβ
	12	11.72 ± 1.86 aγ	15.38 ± 1.29 abγ	18.76 ± 1.33 bγ	26.28 ± 3.12 cγ
	24	11.96 ± 1.57 aγ	23.92 ± 2.15 bδ	31.64 ± 2.05 cδ	31.44 ± 1.60 cδ
Lactic acid	0	0.22 ± 0.16 aα	0.22 ± 0.16 aα	0.22 ± 0.16 aα	0.22 ± 0.16 aα
	6	0.53 ± 0.12 aβ	0.27 ± 0.08 aα	3.65 ± 1.16 bβ	4.64 ± 1.21 bβ
	12	0.31 ± 0.09 aαβ	0.67 ± 0.15 aβ	6.35 ± 1.09 bγ	17.75 ± 1.81 cγ
	24	0.35 ± 0.12 aαβ	2.70 ± 0.13 aβ	12.57 ± 1.28 bδ	9.66 ± 2.04 bδ
Propionic acid	0	0.00 ± 0.00 aα	0.00 ± 0.00 aα	0.00 ± 0.00 aα	0.00 ± 0.00 aα
	6	1.82 ± 0.61 aβ	1.72 ± 0.24 aβ	3.32 ± 1.08 aβ	2.46 ± 0.45 aβ
	12	4.26 ± 0.20 aγ	3.54 ± 0.59 aγ	6.01 ± 0.33 bγ	6.66 ± 1.13 bγ
	24	4.65 ± 0.97 aγ	5.17 ± 0.36 aδ	7.89 ± 0.47 bδ	7.98 ± 1.24 bγ
Butyric acid	0	0.08 ± 0.09 aα	0.08 ± 0.09 aα	0.08 ± 0.09 aα	0.08 ± 0.09 aα
	6	1.61 ± 0.30 aβ	2.33 ± 0.29 aα	1.96 ± 0.62 aβ	1.53 ± 0.52 aα
	12	2.92 ± 0.81 aβ	6.17 ± 0.58 bβ	5.35 ± 0.23 abγ	4.16 ± 1.61abβ
	24	3.17 ± 0.63 aγ	7.98 ± 1.73 bβ	7.75 ± 0.19 bδ	7.50 ± 1.00 bγ

[a] Numbers are means ± standard deviations of triplicate fermentations, expressed as mM.
[b] Total SCFA = acetic acid + lactic acid + propionic acid + butyric acid.
* Different letters (a, b, c, . . .) in the same raw indicate significant differences between different carbohydrates (Tukey test, $p < 0.05$). Different Greek letters (α, β, γ, . . .) for the same short-chain fatty acid in the same column indicate statistical different between different time point (Tukey test, $p < 0.05$).

Vázquez, Charalampopoulos, & Pandiella, 2009). The concentration of lactic acid in FOS increased quickly at first (0.22–17.75 mM) 12 h and decreased later (17.75–9.66 mM), indicating FOS may be fermented quickly at first, and the rate of fermentation into lactic acid was lower than the rate of lactic acid utilized by fecal microbiota later. While fermentation of POS kept increasing the concentration of lactic acid from 0.22 to 12.57 mM, indicating the POS can produce lactic acid for a longer period.

For propionic acid production, once again, POS produced more propionic acid at a higher rate than their parent pectin. Although concentrations of propionic acid increased for all preparations with incubation time, values at 6 h incubation for POS (3.32 mM) are comparable to that of pectin at 12 h (3.54 mM), and values at 12 h (6.01 mM) are higher than pectin at 24 h (5.17 mM). In addition, there is no significant difference between FOS and POS since 12 h incubation.

Butyric acid is the only SCFA that pectin produced more than POS or FOS at all time points, even though the differences were not significant for each time points. This may be due to that butyric acid is not a major end product of *Bifidobacteria* or *Lactobacilli*, but generated mostly by *Clostridia* and *Eubacteria* (Pryde, Duncan, Hold, Stewart, & Flint, 2002), and these two kinds of bacteria both grow well on pectins as we pointed out above.

In this study, the POS generated by DHPM increased the number of *Bifidobacteria* and *Lactobacilli*, and produced a higher concentration of acetic, lactic, and propionic acid than their parent pectin. One the other hand, the number of *Bacteroides* and *Clostridia* was decreased by POS but increased by pectin. These results indicated that the POS was a better candidate of prebiotic than their parent apple pectin. Moreover, the effects of POS on the growth of these bacteria and production of short-chain fatty acids are comparable to those of fructooligosaccharide, further confirmed the potential prebiotic properties of POS. Rastall, Olano-Martin, Mountzouris, and Gibson (2001) manufactured pectic oligosaccharides from commercial pectin by an enzyme membrane reactor, their prebiotic properties were reported better than that of their parent pectins but lower than that of FOS (Olano-Martin, Gibson, & Rastall, 2002). Mandalari et al. (2007) also reported that fermentation of bergamot pectic oligosaccharides resulted in a high increase in the number of *Bifidobacteria* and *Lactobacilli*, whereas the *Clostridia* population decreased, and prebiotic index of POS is even higher than that of FOS. The slight difference among the prebiotic properties found from these studies may be due to the differences in the composition of individual oligosaccharide in these POS. Therefore, researches to investigate the prebiotic properties of individual oligosaccharides and their combined effects would be also necessary and meaningful.

4. Conclusion

The field of prebiotic oligosaccharides is currently a very fertile area for the development of new ingredients and of new products. The successful development of such ingredients will require research advances in the areas of process technology. The dynamic high-pressure microfluidization in this work has been shown to be appropriate for the production of pectic-oligosaccharides from commercial apple pectin. Operational conditions leading to a maximum oligomer production were developed by RSM being pectin concentration 1.84%, DHPM pressure 155 MPa, solution temperature 63 °C and number of cycles 6 passes. Under these conditions, 605.7 mg/100 ml of POS was obtained (yield = 32. 91%).

In the *in vitro* fecal batch culture fermentation, the POS prepared by DHPM increased the number of *Bifidobacteria* and *Lactobacilli*, and produced a higher concentration of acetic, lactic, and propionic acid than their parent pectin. In addition, POS reduced the number of *Bacteroides* and *Clostridia* while their parent pectin increased them. The new oligosaccharides studied here may constitute a good prebiotic candidate, which are comparable to the current market leader, fructooligosaccharides. This study while providing an initial assessment of the prebiotic potential of DHPM-induced oligosaccharides, should be completed by evaluating *in vivo* their fermentation properties before finally used as a functional ingredient for improving the composition of gut microflora.

Acknowledgements

The authors would like to thank the Centre of Analysis and Testing of Nanchang University and State Key Laboratory of Food Science and Technology for their expert technical assistance. This study was supported financially by the National Natural Science Foundation of China (Nr 31260386), and the Research Program of State Key Laboratory of Food Science and Technology, Nanchang University (SKLF-MB-201004).

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