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Research Article

The Promotion Effect of Polysaccharide Isolated from Fresh *tremella* on the Growth of *bifidobacteria*

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Abstract: The current research aimed to evaluate the growth promotion effect of polysaccharide extracted from the fresh *tremella* (TOS) on *bifidobacteria*. Response surface methodology was firstly used to optimize the extraction parameters. The optimum extraction conditions, defined as those resulting in a maximum TOS yield, were liquid (water) material ratio 15 mL/g, temperature 80°C and extraction time 3 h, achieving high polysaccharide extracts yield of 26.41%. The content of carbohydrate and protein from TOS were determined as 0.38±0.02 g/g and 41.70±0.17 mg/g by phenol-sulphuric acid method and Coomassie bluemethod, respectively. The effect of TOS on the growth of *bifidobacterium adolescentis in vitro* was investigated by substituting glucose in the original culture medium with TOS. The results showed that TOS significantly increased the proliferation of *bifidobacterium* compared to glucose at all tested concentration ranging from 0.5 to 16 g/L, while TOS produced a comparable effect on *bifidobacterium* growth to the positive control (Fructooligosaccharides (FOS)) at higher concentration ranging from 4 to 16 g/L. In conclusion, TOS demonstrated a strong propagative effect on *Bifidobacterium* as carbon source.

Keywords: *Bifidobacterium*, fresh *tremella*, polysaccharide extracts

INTRODUCTION

Polysaccharides are polymeric carbohydrate molecules composed of long chains of monosaccharide units linked by glycosidic bonds and on hydrolysis give the constituent monosaccharides or oligosaccharides (Jedrzejas, 2000). Polysaccharides isolated from a range of plants have been identified as prebiotics, which selectively stimulate the growth activity of one or a few colonies of bacteria in the colon, and thus to produce beneficial effects to host health (Rastall and Gibson, 2015). For example, it has been reported that seaweed polysaccharides have the function of correcting intestinal bacterial disorders and inflammatory processes as well as normalizing the immune and metabolic status (Zaporozhets *et al*., 2014). Polysaccharides extracted from *Anoectochilus formosanus* were also found to exhibit the prebiotic effects such as decreasing the cecum pH and increasing calcium absorption and fecal *bifidobacteria* (Yang *et al*., 2012). Besides, evidences also reported that mushrooms are regarded as promising source for prebiotics due to its abundant polysaccharides including chitin, hemicelluloses, α- and β-glucans, mannans, xylans and galactans (Singdevsachan *et al*., 2016). Indeed, pleuran from *oyster* (*Pleurotusostreatus*) mushrooms and lentinan from Shiitake (*Lentinusedodes*) mushrooms are currently the most frequently used β-glucans as prebiotics (Singdevsachan *et al*., 2016).

As one of the most popular cuisine and medicine fungi in China, *tremellafuciformis* (also commonly known as 'snow funguses, 'silver ear' and 'white jelly mushroom') is commercially cultivated (Wen *et al*., 2016). Fruiting body of *tremellafuciformis* are mainly consumed, which are gelatinous, watery white, frond-like, up to 7.5 cm (3.0 in) across (larger in cultivated specimens), and composed of thin but erect, seaweed-like, branching fronds, often crisped at the edges (Pippola and Kotiranta, 2008). *Tremellafuciformis* is traditionally used as a popular nutritious food and a traditional Chinese medicine (Stijve, 2001).

Previous analysis indicated fresh *tremella* comprises 6.7-10% protein, 65-71.2% carbohydrate, 0.6-12.8% fat, 2.4-2.75% crude fiber, 4.0-5.4%

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inorganic salt, 15.2-18.76 % water and a small amount of vitamin B class (Khondkar *et al*., 2002). With the abundant nutrients, *tremella* have a range of biological activities (Du *et al*., 2014). As fungi rich in natural plant gum, *tremella* is beneficial for keeping skin moisture, suppressing wrinkle formation, and promoting wound healing (Khamlue *et al*., 2012; Wen *et al*., 2016). *Tremella* is also a high fiber food, which promotes the gastrointestinal peristalsis and reduces fat absorption (Cheng *et al*., 2002). Furthermore, literatures also indicated that polysaccharides are main bioactive components of *tremella* with anti-oxidant (Ding *et al*., 2011), anti-tumor, anti-diabetic, anti-inflammatory and immune-modulating activity (Wen *et al*., 2011; Du *et al*., 2014). However, due to the limitation of current preserving technology, *tremellafuciformis* is commonly consumed in the form of dried fruiting body. During the drying process, large amount of water-soluble vitamin, glial, carbohydrate and other nutrients in *tremella* will diminish which dramatically reduced the nutrition value of *tremellafuciformis* (Shao *et al*., 2013).

Hence, the objective of the current research was to extract the polysaccharides from the fresh *tremella* by the response surface methodology to explore the optimum extraction conditions; measure their promotion effect on the growth of *Bifidobacterium adolescentis*; and thus evaluate the potential of polysaccharide from fresh *tremella* as a health-promoting diet.

MATERIALS AND METHODS

Material and reagents: Fresh *Tremella* were bought from Fuzhou super market (Fuzhou, Fujian, China). Ethanol, phenol, sulfuric acid, glucose, soluble starch, polymyxin B, sodium acetate and sodium chloride were purchased from the Sigma-Aldrich Chemical Co (St. Louis, MO, USA). Fructooligosaccharides (FOS) was purchased from Bowling Bio-technology Technologies Co., Ltd, Shandong, China. Soy peptone, L-cysteine, yeast extract, and beef extract were purchased from China National Pharmaceutical Group Corporation, China.

Central composite designs: The software Design Expert (8.0.6 trial version, Stat-Ease Inc., Minneapolis, MN, USA) was used for generating the experimental design, data analysis, and model building. Response surface methodology (RSM) has the standard three variables and three levels chosen for Central Composition Design (CCD). The variables and levels chosen for CCD are given in Table 1. The experimental CCD consisted of 17 experimental points which were done in a randomized order. The liquid-solid ratio (A), extraction temperature (B) and extraction time (C) were set as independent variables, while the extraction yield of polysaccharide (Y) was the variable. As shown in Table 2, each experiment in the design matrix was performed in triplicate and the experimental data were obtained.

Table 1: Independent variables and their levels in central composition design (CCD)

	Level		
Variable	-1	0	1
Liquid-solid ratio(g/mL, A)	1:10	1:25	1:40
Extraction temperature (°C, B)	50	65	80
Extraction time (h, C)	1	2.5	4

In order to predict the optimized conditions, a second-order polynomial model was fitted to correlate the relationship between the independent variables and the response (extract yield):

$$Y = \beta_0 + \sum_{j=1}^{k} \beta_j X_j + \sum_{j=1}^{k} \beta_{jj} X_j^2 + \sum\sum \beta_{ij} X_i X_j (i<j)$$

where, Y is the response variables (extract yield); β_0, β_j, β_{jj}, and β_{ij} are the regression coefficients for the intercept, linearity, square and interaction terms; X_i and X_j are the independent coded variables.

Fresh *tremella* polysaccharide extraction: Fresh *tremella* (5g) was mixed with distilled water (the ratio of solid to liquid is 1:10,1:25 and 1:40g/mL, w/v) at different temperature (50,65 and 80°C) for 1,2.5 and4h (Table 1), homogenized (DS-200 electric high-speed tissue homogenizer, Shanghai Precision Instrument, Shanghai, China). The extract was centrifuged and filtered through no.2 filter paper (Whatman International Ltd., Maidstone, Kent, UK), and then concentrated in a rotary evaporator (Rotavapor R-200; Buchi, Postfach, Swizerland). The concentrates were mixed with three volumes of 95% ethanol, and allowed to precipitate overnight. Following centrifugation at 3100 g for 20 min (Hunan Xiangyi Scientific Instrument Corporation, China), the supernatant was removed to obtain the polysaccharides, and then freeze dried to get the fresh *tremella* polysaccharides extract (TOS). The yield of TOS was calculated as followed:

Yield of TOS (w/w) = Weight of dried polysaccharides/Weight of Fresh *tremella*×100%

Determine the carbohydrate content: The total carbohydrate content of the extract was determined by the phenol-sulphuric acid method (Lin *et al*., 2014) with some modification. Briefly, 5 mg sample was hydrolyzed using 0.5 mL of 12 M sulfuric acid for 10 min and then diluted with 2.5 mL distilled water to the final concentration of 2 M sulfuric acid. The sample solution was then further diluted 3-folds using 2 M sulfuric acid. Fifty microliters of phenol solution (5% w/v in water) was added into 50 μL of glucose standards (12.5, 25, 50, 100 and 200 μg/mL in 2 M sulfuric acid), blank solution (2 M sulfuric acid) or sample solution, respectively. 250 μL of 18 M sulfuric

acid was then added rapidly into the solution and vortex-mixed. After 30 minutes reaction, 200 μL of the solution was pipetted into a 96-well plate. The absorbance of solution was measured at 490 nm by a microplate reader (SpectraMax 250, Molecular Devices Corp.USA). Various concentration of glucose was used to plot the standard curve. The carbohydrate concentration of TOS was then calculated according to the standard curve. Total carbohydrate content, expressed as a percentage by weight.

Determine the protein content: The total protein content of the extract was estimated by the Coomassie blue (BioRad, Bradford) method (Melander and Tømmeraas, 2008) with some modifications.

Several of concentrations (1.25, 2.5, 5, 10, 20 and 40 μg/mL) BSA in Milli-Q-water was prepared to obtain the standard curve.1.0 mL of freshly prepared standard solution, Milli-Q water (blank) and various concentration of TOS solution were added into the tested tubes. 0.20 mL of phosphomolybdotungstic reagent were then added and ultrasonicated in order to remove the air bubbles. The solution was mixed and left to stand for 10 min. The absorbance was measured at 750 nm and compared with the calibration curve.

Preparation of *Bifidobacterium adolescentis* stock solution: *Bifidobacterium adolescentis* (Live *Bifdobacterium* Prepa-ration Oral) was bought from Livzon Pharmaceutical Group Inc. (Guangdong, China) and maintained at 4°C. The receipt of growth medium (pH 7.0, 1 liter) included soy peptone (5 g), Glucose (5 g), yeast powder (3 g), beef extract (10 g), soluble starch (1 g), sodium chloride (5 g), sodium acetate (3 g), L-cysteine hydrochloride (0.5 g), polymyxin B (0.02 g). Under sterile conditions, 1 g of freeze-dried bacteria was mixed with 9 mL sterile saline (0.9% m/v). The growth medium (15 mL) was inoculated with the bacteria (16%) and anaerobically incubated in anaero-bic bags (W-zipper Standing-Pouch, Misubishi Gas Chemical, Tokyo, Japan) at 37°C for 48 h. The bacteria were inoculated in the growth medium for 18 h to obtain the bacterial stock solution.

Effect of different concentration of fresh *Tremella* Polysaccharide on the proliferation of *Bifidobacterium*: All fermentation experiments were conducted in a fermentation medium (pH 6.8±0.2), consisting of 10 g/L beef extract, 3 g/L yeast powder, 1 g/L soluble starch, 3 g/L NaAc, 0.02 g/L polymyxin B, 5 g/L carbohydrates, 5 g/L peptone, 5 g/L NaCl, 0.5 g/L l-cysteine hydrochloride. The carbohydrates used in the fermentation medium were glucose, FOSor fresh *tremella* polysaccharide extract (TOS).

The bacterial stock solution was inoculated into the fermentation medium containing glucose, FOS, or TOS as the carbon source at various concentration (0.5, 1.0, 2.0, 4.0, 8.0, and 16.0 g/L) and incubated at 37°C in an anaerobic incubator for 48 h. *Bifidobacterium adolescentis* growth were measured by reading Optical Density (OD) at 600 nm. Each experiment was performed in triplicate.

Statistical analyses: All experiments were performed in triplicates. Data were presented as means±SD. All statistics were performed using GraphPad Prism 5.0 software. Differences were considered to be statistical significant at $p< 0.05$.

RESULTS AND DISCUSSION

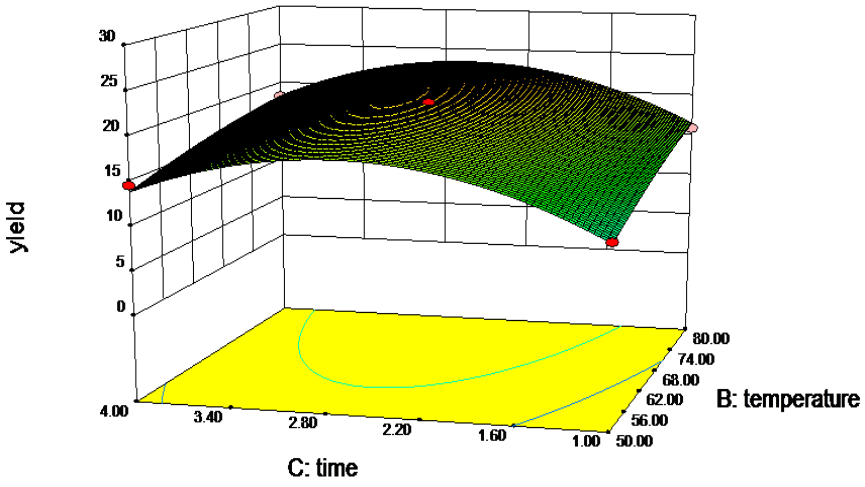
Effect of liquid-solid ratio, temperature and time on extraction yield: The software Design Expert was used to generate the factor response surface analysis diagrams including the 3D response surface stereo analysis diagram as well as the corresponding 2D contour map.

The effects of liquid-solid ratio, temperature and time on the extraction yield of TOS as well as their interactions are shown in Fig. 1. The dependent variable showed a complicated correlation with the independent variables. The extraction yield values ranged from 1.97-21.37% (w/w, based on the weight of fresh *Tremella*).

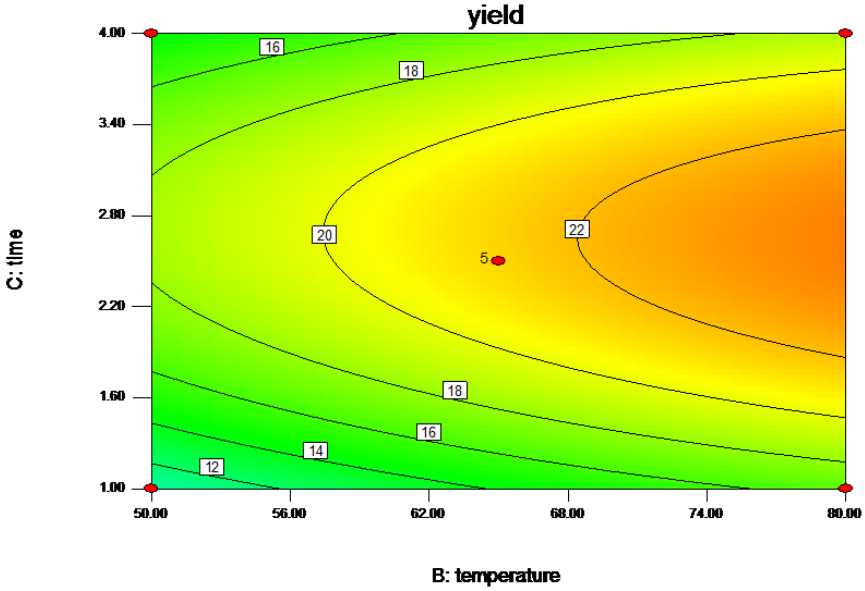
The 3D plot and the contour plot in Fig. 1a and 1b showed the interaction effect of extraction time and extraction temperature on the yield of TOS. The extract yield showed an increasing trend within the temperature range from 50 to 80°C, which indicated temperature, had a positive impact on the extraction yield of TOS. It has been reported that temperature may cause the mass transfer of the extract (Ji *et al*., 2006). Regarding the effects of extraction time on the yield, although Qiao *et al*. (2009) reported that a longer extraction time favored the extraction efficiency for polysaccharides, however, in the current research, the extraction yield increased at the initial stage and then slightly decreased with the extension of the extraction time, suggesting excessive prolonged extraction time may also negatively affected the polysaccharides yield.

Figure 1c and 1d showed the interactive effects of liquid-solid ratio and extraction temperature on the fresh *tremella* polysaccharide extraction yield. Under the same extraction temperature, yield of fresh *tremella* polysaccharide increased up to a summit and then decreased with the increase of liquid-solid ratio; fresh *tremella* polysaccharide yield increases with the extraction temperature under the same liquid-solid ratio. When the ratio of liquid-solid is 1:10-1:25 and extraction temperature is located between 62-80°C, the fresh *tremella* polysaccharides extraction yield reached a maximum.

Figure 1e and 1f showed when the extraction temperature is 65°C, the liquid-solid ratio and extraction time affected the fresh *tremella* polysaccharide yield. The results demonstrated fresh


yield
30
25
20
15
10
5
0
4.00
3.40
2.80
2.20
1.60
1.00
C: time
80.00
74.00
68.00
62.00
56.00
50.00
B: temperature

(a)


yield
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C: time
16
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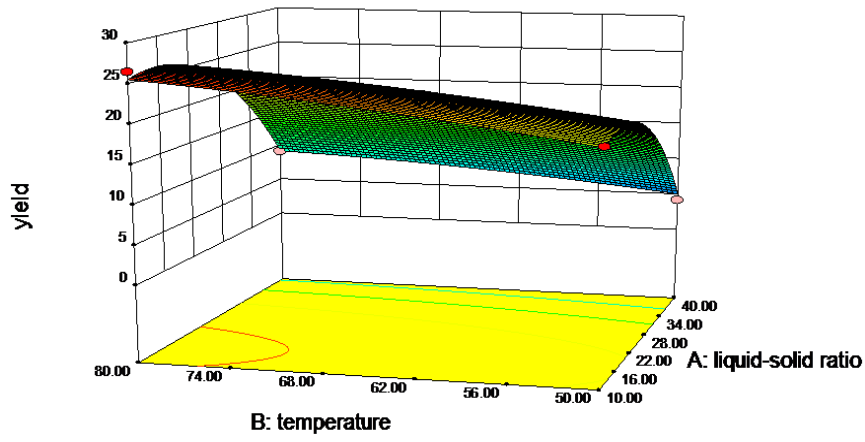
62.00
68.00
74.00
80.00
B: temperature

(b)


yield
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B: temperature
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28.00
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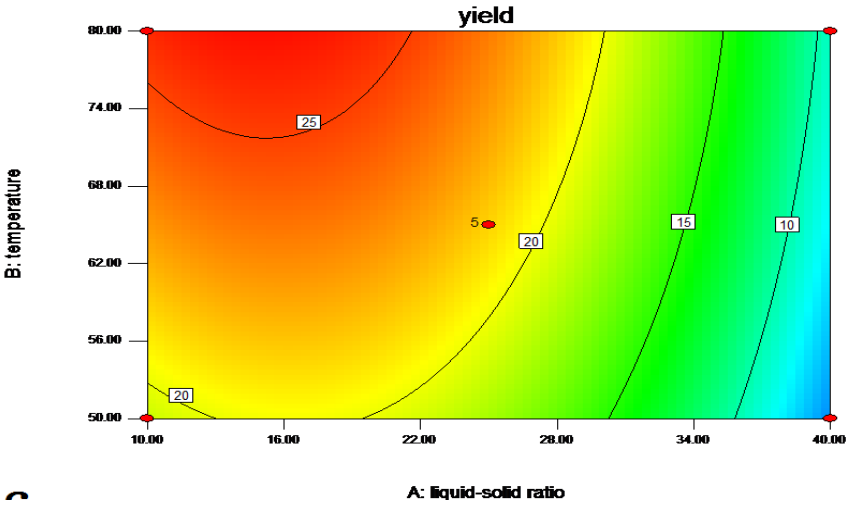
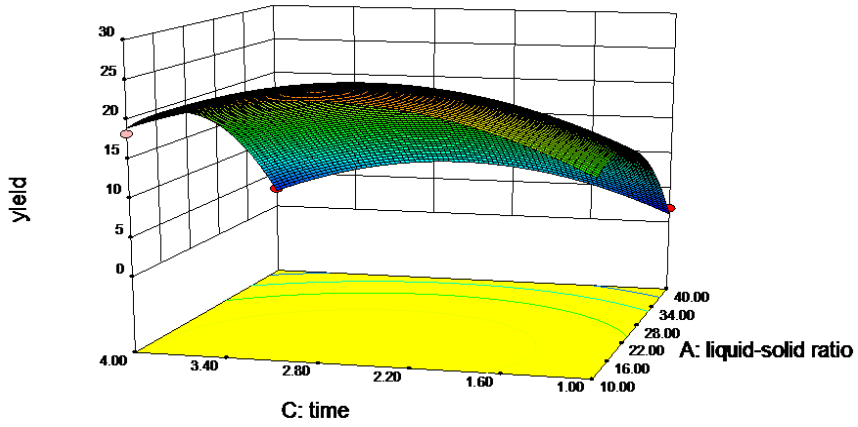
A: liquid-solid ratio

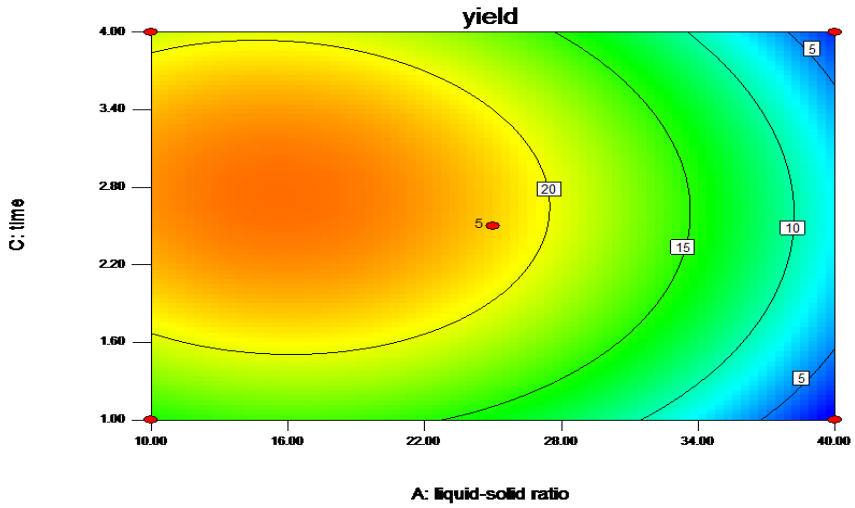
(c)



(d)



(e)



(f)

Fig. 1: Response surface plot showing the effects of extraction temperature (B) and extraction time (C) (a&b); liquid-solid ratio (A) and extraction temperature (B) (c&d); and liquid-solid ratio (A) and extraction time (C) (e&f) on the yield of TOS

Table 2: Experimental design and results for extraction yield of TOS (n = 3)

Run	A (g/mL)	B (°C)	C (h)	Yield of extract (%)[a]
1	1	0	1	2.56
2	-1	-1	0	19.34
3	0	1	-1	15.9
4	-1	1	0	26.5
5	0	0	0	21.37
6	1	-1	0	4.21
7	-1	0	1	18.24
8	0	1	1	18.3
9	1	1	0	9.15
10	1	0	-1	1.97
11	0	0	0	21.37
12	0	0	0	21.37
13	0	-1	1	14.61
14	0	-1	-1	10.7
15	-1	0	-1	14.73
16	0	0	0	21.37
17	0	0	0	21.37

[a]Each value is the mean of triplicate measurements

Table 3: Analysis of variance for the experimental results of the TOS

Source	Sum of squares	Degrees of freedom (df)	Mean square	*F*-value	*P*-value probe>*F*
Model	862.30	9	95.81	170.28	<0.0001
A: liquid-solid	463.91	1	463.91	824.46	<0.0001
B: temperature	55.07	1	55.07	97.88	<0.0001
C: time	13.55	1	13.55	24.07	0.0017
AB	1.23	1	1.23	2.19	0.1825
AC	2.13	1	2.13	3.79	0.0927
BC	0.57	1	0.57	1.01	0.3477
A^2	153.42	1	153.42	272.65	<0.0001
B^2	1.20	1	1.20	2.13	0.1876
C^2	149.50	1	149.50	265.70	<0.0001
Residual	3.94	7	0.56		
Lack of Fit	3.94	3	1.31		
Pure Error	0.000	4	0.000		
Cor Total	866.24	16			

tremella polysaccharide yield gradually increased before a decline occurred with the extension of extraction time under the same liquid-solid ratio; similarly, fresh *tremella* extract yield displayed an upward trend and then decreased with increase of liquid-solid ratio at the same extraction time. When the liquid-solid ratio is between 1:10 and 1:20 and the extraction time was 1.5-3.5 h, the fresh *tremella* polysaccharide yield is largest.

Model fitting: The values of the responses for different experimental conditions are presented in Table 2, which demonstrated that a considerable variation was incurred in the yield values of TOS within the range of the extraction conditions. Using the software Design Expert, a polynomial model describing the correlation between the yield and the three variables were obtained as follows:

$$Y = 21.37 - 7.62A + 2.62B + 1.30C - 0.56AB - 0.73AC - 0.38BC - 6.04A^2 - 0.53B^2 - 5.96C^2$$

where, Y represents the extraction yield, and A, B and C are the liquid-solid ratio, extraction temperature and extraction time, respectively. The *F*-test and *P*-value were used to measure the significance of the coefficients of the model. The results were summarized in Table 3. The analysis of variance (ANOVA) of the quadratic regression model demonstrated that the model was highly significant (p<0.0001) and the result suggested that the model is adequate for predicting within the range of the variables employed.

Besides, the *p*-values for the extraction yield of TOS were highly significant, which indicated that the fitness was significant, while the lack-of-fit values showed that the lack-of-fit were not significant relative to the pure error.

In addition, the correlation coefficient indicated that the regression models defined the behavior of the system. Determination coefficient (R^2) is defined to be the ratio of the explained variation to the total variation and is a measurement of the degree of fitness. The model can fit well with the actual data when R^2 approaches unit. By analysis the variance, the R^2 value was 0.9955 for the extraction yield of TOS (Table 4), which proved that the regression model defined well the true behavior of the system. The adjusted R^2 value (0.9896) is also very high, making the model very significant. The Coefficient of Variation (CV) indicates

Table 4: The R2, Adj R2 and CV values of the response surface quadratic model for the yield of TOS

Values	R^2	Adj R^2	Coefficient of the variation (CV) (%)
Extraction yield	0.9955	0.9896	4.85

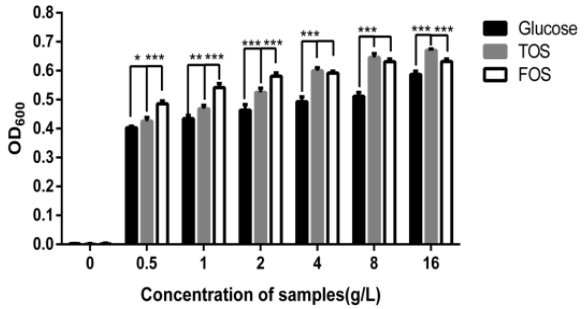


Fig. 2: Effect of fresh *tremella* polysaccharides extract at different concentrations on the proliferation of *Bifidobacterium adolescentis in vitro*. Data are presented as mean±SEM, n = 3. **p*<0.05, ***p*<0.01, ****p*<0.001 by one-way ANOVA followed by Tukey's multiple comparison

the degree of precision with that the treatments are compared. Usually, the higher the value of the CV, the lower is the reliability of the experiment. The CV value in the current research was analyzed to be 4.85%. All of these results indicated that the polynomial model equation had a high quality fit, a good precision and reliability within the range of the independent variables used.

Optimization for extraction of TOS: The analysis of response surface was performed by Design Expert software to determine the optimal extraction conditions. The optimal extracting conditions of TOS were liquid (water) material ratio 14.74 mL/g, temperature 80°C and extraction time 2.68 h, respectively. Under these conditions, the predicted yield of GLP was 26.31%.

The suitability of the model equation for predicting the optimum response values was tested by using the selected optimal conditions. Rounding these numbers, the optimal parameters were chosen as: liquid (water) material ratio 15 mL/g, temperature 80°C and extraction time 3 h. Under these parameters, the real experimental yield was 26.41 %. There was no significant difference between the predicted value and experimental value (p>0.05). The result suggested that the regression model was accurate and adequate for the prediction of TOS extraction.

Composition analyses: After establish the standard curves of Y = 6.494X+0.009 (R^2 = 0.9965) and y = 0.4391x-0.0144 (R^2 = 0.9918) using a dilution series of glucose standards and protein standards (BSA), the carbohydrate content in the TOS is determined as 0.38±0.02 g/g extract while the protein content was 41.70±0.17 mg/g, indicating that the extract contained abundant polysaccharide.

Effect of different concentration of fresh *Tremella* Polysaccharide on the proliferation of *Bifidobacterium adolescentis*: After 48 h of fermentation in the medium containing different carbon sources at various concentrations, the amount of *Bifidobacterium adolescentis* (by measuring absorbance at 600 nm) was shown in Fig. 2.

The increase in all three carbon source led to a clear upward trend of *Bifidobacterium adolescentis* proliferation, suggesting within a certain range, carbon source rich medium may be more suitable for rapid growth of *Bifidobacterium adolescentis.* Furthermore, compared to monosaccharides glucose, TOC exerted a significant stronger promotion effect on *Bifidobacterium adolescentis* growth at all tested concentration ranging from 0.5 to 16 g/L. This promotion effect became more pronounced at high concentrations. When the concentration was higher than 4 g/L, TOC produced a comparable effect on *Bifidobacterium* growth to the positive control (oligofructose). At 16 g/L, the effect of TOS on *Bifidobacterium adolescentis* proliferation was even significantly higher. These results together indicated that TOS maybe a carbon source to support rapid growth for *Bifidobacterium adolescentis*.

CONCLUSION

In this study, the fresh *tremella* was used as raw material to extract TOS by the hot water. Based on the results from response surface experiments, the optimal extraction conditions were established as followed: solid to liquid ratio 15 mL/g, extraction temperature 80°C, extraction time 3 h. using these parameters, we successfully obtained the purified polysaccharides from *Bifidobacterium adolescentis* with high yield, high purity and low proteins contaminants. The purified polysaccharides were approximately achieved at the yields of around 26.41% (w/w) of raw fresh *tremella* weight. The composition analysis further indicated the purity of polysaccharides reached at around 40% with small amount of proteins contaminations at around 0.4%.

Furthermore, we also tested the effects of polysaccharides extracted from *tremella* on *Bifidobacterium adolescentis* growth. Results showed that both TOS and FOS have stronger propagative effect on *Bifidobacterium* compared to glucose, suggesting polysaccharides may accelerate *Bifidobacterium adolescentis* growth compared to glucose and monosaccharides. Besides, the results also highlighted the potential of polysaccharides isolated from *tremella* to be used as health-promoting diet to mediate the gastrointestinal tract ecosystem.

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