Carbohydrate Polymers 111 (2014) 775–782

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

# Optimization of enzyme assisted extraction of *Fructus Mori* polysaccharides and its activities on antioxidant and alcohol dehydrogenase

Qingfang Deng[a,b], Xin Zhou[a,b,*,1], Huaguo Chen[a,b,**,1]

[a] *Key Laboratory for Information System of Mountainous Areas and Protection of Ecological Environment, Guizhou Normal University, 116 Baoshan North Road, Guiyang, Guizhou 550001, PR China*
[b] *The Research Center for Quality Control of Natural Medicine, Guizhou Normal University, 116 Baoshan North Road, Guiyang, Guizhou 550001, PR China*

ARTICLE INFO

*Article history:*
Received 14 April 2014
Received in revised form 7 May 2014
Accepted 14 May 2014
Available online 22 May 2014

*Keywords:*
*Fructus Mori* polysaccharides
Enzyme assisted extraction
Antioxidant activity
Activate alcohol dehydrogenase activity
Response surface methodology

ABSTRACT

In the present study, enzyme assisted extraction of *Fructus Mori* polysaccharides (*FMPS*) from *F. mori* using four kinds of enzymes and three compound enzymes were examined. Research found that glucose oxidase offered a better performance in enhancement of the extraction yields of *FMPS*, antioxidant and activate alcohol dehydrogenase activities. The glucose oxidase assisted extraction process was further optimized by using response surface method (*RSM*) to obtain maximum yield of crude *FMPS*. The results showed that optimized extraction conditions were ratio of enzyme amount 0.40%, enzyme treated time 38 min, treated temperature 58 °C and liquid–solid radio 11.0. Under these conditions, the mean experimental value of extraction yield (16.16 ± 0.14%) corresponded well with the predicted values and increased 160% than none enzyme treated ones. Pharmacological verification tests showed that *F. mori* crude polysaccharides had good antioxidant and activate alcohol dehydrogenase activities in vitro.



## 1. Introduction

*Morus alba* L. are large, deciduous trees native to tropical, temperate, sub-arctic, and subtropical regions, and they mainly concentrate in China, Japan and India in Asia (Nomura & Fukai, 1981). The fresh fruit of *Morus alba*, also called mulberry fruit, has been well accepted owing to its delicious, low in calories and rich in a variety of nutrients (Abbasi, Khan, Khan, & Shah, 2013). *Fructus Mori*, the dried mulberry fruit, has been used as a tonic and sedative for several thousand years in traditional Chinese medicine, to prevent diabetes and other various chronic diseases (Kim et al., 2011).

In recent years, further researches on chemical components and pharmacologic effects of *F. Mori* found that it contains many bioactive constituents, such as carbohydrates, flavonoids, alkaloid, amino acid, vitamin, etc. (Yoshinaga et al., 2007) and mulberry extract has been reported to have not only the anti-oxidative or radical-scavenging property, anti-inflammatory, immunizing activity, anti-tumor, anti-diabetic effects, anti-aging property, and liver protection (Jiang et al., 2013; Tang, Huang, Lee, Tang, & Wang, 2013), but also beneficial effects on cardiovascular, neuroprotective properties, hypoglycemic and atherosclerosis (Kim, 2010).

*F. Mori* polysaccharides (*FMPS*), one of the biological active ingredients isolated from *F. Mori*, was proved to have the function of anti-aging property-enhance immunity, anti-inflammatory (Liu & Lin, 2012), hypoglycemic and hypolipidemic effect (Tian, Bo, & Li, 2011) with no toxic record in clinic. Besides, studies have shown that many polysaccharides including *FMPS* have antioxidants activities (Wang, Chang, Inbaraj, & Chen, 2010), but the researches on the antioxidants activities of *FMPS* are insufficient. In this paper, the antioxidants activities of polysaccharides obtained from *F. Mori* were investigated by the scavenging activity of the 2,2-diphenyl-1-picrylhdrazyl (*DPPH*) radical. Moreover, *F. Mori* was popular used in China as an important dealcoholic drug. Ethanol is mainly metabolized in liver by ethanol dehydrogenase (*ADH*), cytochrome P450-2E1 and acetaldehyde dehydrogenase. Ethanol

* Corresponding author at: The Research Center for Quality Control of Natural Medicine, Guizhou Normal University, 116 Baoshan North Road, Guiyang, Guizhou 550001, PR China.
** Corresponding author at: Key laboratory for Information System of Mountainous Areas and Protection of Ecological Environment, Guizhou Normal University, 116 Baoshan North Road, Guiyang, Guizhou 550001, PR China.
Tel.: +86 851 18085071273.
*E-mail addresses:* alice9800@sina.com (X. Zhou), huaguochen81@gmail.com (H. Chen).
[1] These authors contribute equally to the work.

http://dx.doi.org/10.1016/j.carbpol.2014.05.018
0144-8617/

metabolic enzyme plays an important role in the metabolism of ethanol (Lee, Wang, Lee, & Yin, 2003). So the ADH activities of *FMPS* were investigated in this study.

It has been widely acknowledged that bioactivity activities of polysaccharides can be affected by many factors including its chemical components, molecular mass, structure, and conformation even the drying methods (Edwards et al., 2014; Francisco, Franco, Wagner, & Jacob-Lopes, 2014). However, there is no information about the effect of extraction methods on antioxidants activities and *ADH* activities of polysaccharides from *F. Mori*. And enzyme assisted extraction as an effective and advisable emerging technology in the food industry may be a better extraction of polysaccharides. So in order to seek the suitable enzyme for the production of active polysaccharides, antioxidants activities and *ADH* activities were used to evaluate *FMPS* acquired by none enzyme extraction, four single enzyme extraction with four different enzymes ($\beta$-dextranase, pectinase, cellulase, Glucose oxidase (*GOD*)) and three compound enzymes extraction ($\beta$-dextranase: pectinase, $\beta$-dextranase: *GOD*, $\beta$-dextranase: pectinase: *GOD*).

Response surface methodology (*RSM*), as an effective statistical method, is widely used for conditions of enzyme reaction, optimizing complex process, extraction technology, and so on. Since it can depict the complete effects of variables, evaluate the interactions between multiple parameters, reduce the number of experimental trials and shorten working time. It is more precise and effective than many approaches (Hosseinpour, Vossoughi, & Alemzadeh, 2014; Kumar, 2013). Therefore, in this paper, a three-level, four-variable (enzyme treated time–liquid–solid ratio, enzyme amount and enzyme treated temperature) Box–Behnken design (BBD) of RSM was employed to further optimize the approach of glucose oxidase assisted extraction conditions for *FMPS*.

## 2. Materials and methods

### 2.1. Materials and reagents

*F. Mori* was purchased from Tong Ren Tang Co., Ltd. (China). Cellulose and $\beta$-dextranase were obtained from Sukahan Bio-Technology Co., Ltd. (China). Pectinase was acquired from Jatou Industrial & Commercial Co., Ltd. (China). Glucose oxidase was bought from Sigma–Aldrich (USA). 2,2-Diphenyl-1-picrylhdrazyl (*DPPH*) was purchased from Aladdin Chemistry Co. Ltd. (China). Alcohol dehydrogenase (ADH) and $\beta$-nicotinamide adenine dinucleotide ($NAD^+$) were obtained from Heowns Biochem Technologies LLC (China). All other chemicals and solvents used were of analytical grade.

### 2.2. Preparation of F. mori

*F. Mori* was ground by grinder machine to obtain a fine powder. The powders of *F. Mori* were degreased with 80% ethanol at 60 °C for three times (2 h each time) by reflux, and then residues were removed some colored materials with aether by soxhlet extraction. The pretreated samples were dried by vacuum dryer (50 °C).

### 2.3. Extraction of crude F. mori polysaccharides (CFMPS)

Calculated amounts of the above 4 enzymes were weighed, and distilled water was added to obtain stock solutions at the concentration of 3 mg $mL^{-1}$, respectively. The stock solutions were further diluted with distilled water and adjusted with acetate buffer to obtain serial solutions with desired concentration and pH value.

Different extractions from the *F. Mori* were carried out as detailed below. Each dried preprocessed sample (20 g) was mixed with 0.15% $\beta$-dextranase, 0.15% pectinase, 0.15% cellulase, 0.15% glucose oxidase, 0.15% compound enzymes 1 ($\beta$-dextranase and glucose oxidase; 1:1), 0.15% compound enzymes 2 ($\beta$-dextranase and pectinase; 1:1), 0.15% compound enzymes 3 ($\beta$-dextranase, pectinase and glucose oxidase; 1:1:1), respectively. The mixtures were extracted by water in a designed extraction temperature (50 °C), extraction time (2 h), and liquid–solid ratio 10 (v/w). The water extraction solutions were separated from insoluble residue through vacuum filter, and concentrated in a rotary evaporator under reduced pressure, and then precipitated by adding 95% ethanol to a final concentration of 80% (v/v) (for 24 h at 4 °C), the collected precipitate was removed protein by the Sevag method (Cohen & Johnstone, 1964) and dried under reduced pressure at 50 °C to acquire crude polysaccharides until its weight was constant. Crude polysaccharide was weighted with an electronic balance (AL204, Mettler Toledo instrument co., LTD, Shanghai, China). The yield of the crude polysaccharides extract (%) was calculated as follows:

$$\text{Yield of crude polysaccharides extract}(\%) \left(\frac{W}{W}\right) = \frac{\text{dried crude extraction weight(g)}}{\text{powder weight(20 g)}} \times 100 \quad (1)$$

The polysaccharide content of precipitate was measured by the phenol–sulfuric acid method (Saha & Brewer, 1994) using D-glucose as a standard on Varian 100 UV/Visible spectrophotometer. The *CFMPS* yield (%) was calculated using the formula as follows:

$$\text{The CMFPS yield}(\%) = \frac{C}{W} \times 100 \quad (2)$$

where $C$ is the content of polysaccharides in the concrete and $W$ represents the dry sample weight (20 g).

### 2.4. Determination of antioxidant activities of CFMPS

Antioxidants have abilities to seize the free-radical chain of oxidation and form stable free radicals, which would prevent further oxidation. *DPPH* has been used extensively as free radical to evaluate reducing substances. In this paper, the *CFMPS* were evaluated for their abilities to scavenge *DPPH* radical using a modified protocol based on Yang, Zhao, Shi, Yang, and Jiang (2008). Briefly, 2 mL different concentrations of the *CFMPS* solution were added to 2 mL *DPPH* solution (0.2 mM in anhydrous ethanol). After shaking vigorously, the mixture was incubated at 25 °C in the dark for 40 min. The absorbance ($A$) was measured at 517 nm against a blank and a control, distilled water ($A_0$) and anhydrous ethanol ($A_1$) instead of the *CFMPS* solution and *DPPH* solution, respectively. The scavenging activity of the *DPPH* radical was determined using the following equation:

$$\text{DPPH radical scavenging activity}(\%) = \left[1 - \frac{A - A_1}{A_0}\right] \times 100 \quad (3)$$

### 2.5. Assay of alcohol dehydrogenase activity

The effect of *CFMPS* on activity of alcohol dehydrogenase was determined by the Valle & Hoch method with modification (Vallee & Hoch, 1955). Briefly, 0.2 mL different concentrations of the *CFMPS* solution were added to 1.5 mL sodium pyrophosphate solution (0.1 M), 0.5 mL ethanol (2.0 M) and 1.0 mL $NAD^+$ solution (0.025 M). After shaking vigorously, the mixture was incubated at 25 °C for 2–3 min to achieve temperature equilibrium and establish blank rate (distilled water instead of the *ADH* solution). At zero time, add 0.1 mL of befittingly diluted *ADH* to the cuvette and recorded the rate of absorbance at 340 nm resulting per 30 s for 5 min immediately measuring the increase of *NADH* absorption as function of time by Varian 100 UV/Visible spectrophotometer. Calculate the $\Delta A_{340}$/min from the initial linear portion of the curve, and the *ADH* activity has been expressed in the units recommended by the

**Table 1**
Effect of different enzymes on *CFMPS* content, scavenging rate of *DPPH* radical and activities of *ADH*.

| Extraction method | Extraction yield (%) | DPPH radical scavenging ability (%) | ADH activation rate (%) |
|---|---|---|---|
| None enzyme extracts | 9.21 | 82.64 | −7.68 |
| Cellulase extracts | 11.69 | 85.81 | 168.89 |
| Dextranase extracts | 14.65 | 87.27 | 180.00 |
| Pectinase extracts | 14.01 | 85.86 | 91.11 |
| GOD extracts | 15.23 | 91.99 | 301.11 |
| Compound enzymes 1 extracts | 13.23 | 56.6 | 1.01 |
| Compound enzymes 2 extracts | 12.85 | 51.14 | 313.33 |
| Compound enzymes 3 extracts | 13.56 | 90.85 | 256.67 |

book of the Bio-chemical. The inhibitory (activation) rate of *ADH* ($U\,mL^{-1}$) was calculated as follows:

$$\text{Inhibitory(activation) rate}(\%) = \frac{NC - PC}{NC} \times 100 \quad (4)$$

where *NC* is the *ADH* activity of negative control and *PC* represents the *ADH* activity of positive control.

### 2.6. Experimental design

After determining the species of enzyme through the bioactivities and yields of *CFMPS*, the single-factor test was used for obtaining the preliminary range of extraction variables, then a three-level-four-factor BBD of RSM was used to determine the optimal combination of extraction variables. Based on the results of single factor experiments, four independent variables were enzyme treated time ($X_1$, min), ratio of enzyme amount ($X_2$, %), liquid–solid ratio ($X_3$, v/w) and enzyme treated temperature ($X_4$, °C), while the dependent variable (response variable) was the extraction yield of *CFMPS* (%). Each variable was designated as three levels, coded +1, 0 and −1 for high, intermediate and low value, respectively. The response could be related to the selected variables by the following second-order polynomial model:

$$Y = \sum A_0 + \sum_{i=1}^{k} A_{ij}X_i + \sum_{i=1}^{k} A_{ii}X_i^2 + \sum_{i=1}^{k-1}\sum_{j=i+1}^{k} A_{ij}X_iX_j \quad (5)$$

where $Y$ is the response variable, $A_0$, $A_i$, $A_{ii}$ and $A_{ij}$ are the regression coefficients for intercept, linear, quadratic and interaction terms, respectively. $X_i$ and $X_j$ are the encoded independent variables ($i \neq j$) affecting the response of $Y$.

### 2.7. Statistical analysis

SPSS 18.0 software (SPSS Inc., Chicago, IL, USA) was used to analyze the bioactivity experimental and single-factor experimental data. The experimental design and the regression analysis of experimental data exploited Design-Expert 8.0.5 (Trial version, Stat-Ease Inc., Minneanopolis, MN, USA).

The regression coefficients were performed in the form of analysis of variance (*ANOVA*). Student's *t*-test was employed for evaluating the statistical significance of the regression coefficient, and Fischer's *F*-test at a probability ($P$) of 0.001, 0.01 or 0.05 was used to determine the second-order model equation and its fitness was expressed by the regression coefficients $R^2$. The adequacy and significance of the model was tested by *F*-value, determination coefficient ($R^2$) and lack of fit secured from *ANOVA*.

## 3. Results and discussion

### 3.1. Selection of enzyme type

It is well-known that the plant cell wall is three-dimensional dynamic networks composed of cellulose, pectin crosslinking glycans and proteins (Pauchet et al., 2014). Pectinase can break down pectic compounds and pectin. Beta-dextranase has the ability to disintegrate the $\beta$-glucans. Cellulase catalyzes can decompose the cellulose into cellobiose, glucose and higher glucose polymers. Glucose oxidase can catalyze the formation glucose acid and hydrogen peroxide by disintegrating $\beta$-D-glucose in the presence of oxygen.

In this study, aqueous enzymatic extraction of *CFMPS* was carried out by four enzymes with single enzyme experiments and compound enzymes experiments. As shown in Table 1, the extraction yield of *CFMPS* by different extraction methods listed in the order of glucose oxidase extract > pectinase extract > compound enzymes 3 extract > compound enzymes 2 extract > $\beta$-dextranase extract > compound enzymes 1 extract > cellulase extract > none enzyme extract, and was 14.23%, 14.01%, 13.56%, 13.23%, 13.08%, 12.85%, 11.69% and 9.21%, respectively. The glucose oxidase extract had the highest content of polysaccharides while none enzyme extracts had the lowest extraction yield.

Table 1 also describes the scavenging abilities of *CFMPS* on the *DPPH* radical. The *DPPH* scavenging ability decreased from 91.99% to 51.14% in the following order: glucose oxidase extract > compound enzymes 3 extract > $\beta$-dextranase extract > pectinase extract > cellulase extract > none enzyme extract > compound enzymes 1 extract > compound enzymes 2 extract. These results suggested that the scavenging activities of enzymes extracts obtained by different methods had significant differences at the same concentration of $2.0\,mg\,mL^{-1}$. And the glucose oxidase extract had the strongest scavenging power on *DPPH* radical in contrast to the weakest one of the compound enzymes 2 extract. In addition, there were more than 40% *DPPH* scavenging ability between the highest one and the lowest one.

The activated effects of *CFMPS* on the *ADH* were measured and shown in Table 1. The extracts obtained through single enzyme extractions and compound enzymes extractions had significantly different activation rates of *ADH* while none enzyme extraction had inhibition ratio of ADH (−7.68%) at the same concentration point ($2\,mg\,mL^{-1}$). Among the four single enzyme extracts, glucose oxidase extract had the highest *ADH* activation rate (301.56%) relative to the lowest one (91.32%) of pectinase extract. Among the three compound enzymes extracts, compound enzymes 2 extract as the strongest one to *ADH* was 56.73% higher than compound enzymes 3 extract, while compound enzymes 1 extract had done little to *ADH*. Certainly we can find that the extracts obtained by glucose oxidase extraction showed obviously stronger activation than that of extracts obtained using other methods. No significant difference ($p > 0.05$) was

found between glucose oxidase extract and compound enzymes 2 extract.

These results showed that the extracts obtained from *F. Mori* using different extracting methods exhibited different extraction yields, antioxidant activities and *ADH* activities. Among the seven extracts, glucose oxidase extract had the highest yield of polysaccharides, highest scavenging effects on *DPPH* radical, and the stronger abilities on *ADH*. Glucose oxidase was proved the best performer among the enzymes tested. Therefore, glucose oxidase was chosen as the optimal enzymes in the present work.

### 3.2. Effect of enzyme treated temperature on yield of CFMPS

It is very important for *CFMPS* extraction to keep glucose oxidase at an optimal working temperature. In the present study, the effect of various enzyme treated temperature points within 25–85 °C used on extraction of *CFMPS* was investigated, while keeping the treatment time, liquid–solid radio and enzyme amount constant at 120 min, 12 and 0.30%, respectively. The *CFMPS* content in extract increased from 8.84% to 13.30% with the increase of treatment temperature from 25 to 55 °C, and peaked at around 55 °C. Further enhancing of temperature, however, resulted in decreasing *CFMPS* content in extract to 10.88% at 85 °C. Thus, the temperature chosen for *CFMPS* extraction was 55 °C.

### 3.3. Effect of enzyme amount on yield of CFMPS

Enzyme amount is also an important factor for *CFMPS* extraction from plant materials. Too small, more time will be taken up and polysaccharides in raw material cannot be completely enzyme treated. Too high, it will cause high process cost. The effect of enzyme amount on extraction yield was investigated in this study. Increasing in the tested ratio of enzyme to raw material (from 0.10% to 0.30%) could improve *CFMPS* extraction from 12.64% to 14.37%, and the increase leveled off at ratio of 0.30%. As the results of statistical analysis showed that significant differences were for the enzyme amount tested ($P < 0.05$). Therefore an optimal ratio of 0.30% was favorable for polysaccharides production.

### 3.4. Effect of liquid–solid ratio on yield of CFMPS

To investigate the effect of liquid–solid ratio on extraction yield of *CFMPS*, a liquid–solid ratio range from 6 to 18 was tested while the treatment time, temperature and enzyme amount were kept at 60 min, 55 °C and 0.30%, respectively. An increase of *CFMPS* content was observed with the increase of liquid–solid ratio from 6 to 12, reaching the highest at liquid–solid radio 12 (*CFMPS* content equals to 15.59%). Therefore, liquid–solid ratio 12 was chosen as the optimal one in the present experiment.

### 3.5. Effect of enzyme treated time on yield of CFMPS

Treatment time is a very important factor for enzymatic activity and to be considered. It will have an effect on the final yield of CFMPS in the abstraction, the energy cost and the efficiency of extraction. In this study, an increase of *CFMPS* extraction from 14.58% to 15.76% was observed with the elevation of treatment time from 15 min to 60 min, reaching the highest value at 60 min, but further increase of enzyme treated time resulted in decreasing *CFMPS* content to 13.74% at 120 min. Thus, the treatment time 60 min was chosen as the optimal one based on the study.

### 3.6. Optimization of extraction conditions of CFMPS

#### 3.6.1. Statistical analysis and the model fitting

The *BBD* of *RSM* in the experimental design involves four independent variables, three levels and five replicates at the center point (Table 2), which were carried out to measure the inherent variability and process stability. The experimental conditions and the fit statistics of extraction yield of 29 runs with BBD design were shown in Table 2, and all tests were performed in triplicate. As shown in Table 2, the extraction yield of *CFMPS* values (%) varied from 12.93 to 15.96%.

The results of extraction yield affected by enzyme treated time, enzyme amount, liquid–solid ride and enzyme treated temperature were fitted to a second-order polynomial equation, and the values of regression coefficients were calculated.

The effects of four variables were highly significant on extraction yield of *CFMPS* (Table 3). The predicted model of the extraction yield value was obtained by the following second-order polynomial equations:

$$Y_1 = 15.88 - 0.31X_1 - 0.32X_2 + 0.38X_3 + 0.19X_4 + 0.81X_1X_2 + 0.14X_1X_4 - 0.10X_1X_4 - 0.46X_2X_3 + 0.20X_2X_4 + 0.90X_3X_4 - 0.90X_3X_4 - 0.74X_1^2 - 0.85X_2^2 - 0.66X_3^2 - 1.17 \quad (6)$$

The predicted values of extraction yield based on the above quadratic predictive model were shown in Table 2.

The statistical significance of regression equation was evaluated by *F*-test, *T*-test and *AVNOA* for response surface quadratic polynomial model were presented in Table 3. The results of high model *F*-value (594.59) and low *P*-value ($P < 0.0001$) turned out that the models were highly significant. The determination coefficient ($R^2$) for model (0.9983) was close to 1.0, which represented the satisfactory correlation between actual and predicted values. The value of adjusted determination coefficient $R^2$ (Adj. $R^2$) value was 0.9966, which meant most variation (>99%) of the extraction yield could be predicted by the model, and less than 1% variations could not be explained by the model.

The lack-of-fit used to measure the failure of the model to represent the data in the experimental domain at points which were not included in the regression. The *F*-value of 0.19 and *P*-value of 0.9847 for extraction yield implied that the lack of fit was not significant relative to the pure error due to noise. Adequate precision compared the range of the predicted values at the design points to the average prediction error. The ratio greater than 4 indicated adequate model discrimination. In this research, the values were well above 4.

The *P*-values were used as a tool to check the significance of each coefficient, which in turn may indicate the pattern of the interactions between the variables. The smaller the value of *P* was, the more significant the corresponding coefficient was. It can be seen from Table 3 that linear coefficients ($X_1$, $X_2$, $X_3$, $X_4$), quadratic term coefficient ($X_1^2$, $X_2^2$, $X_3^2$, $X_4^2$) and cross product coefficients ($X_1X_2$, $X_2X_3$, $X_2X_4$, $X_3X_4$) were very significant with *P* values ($P < 0.01$). The other term coefficients were significant ($P < 0.05$).

#### 3.6.2. Analysis of response surfaces

The 3D response surface and 2D contour plots were the graphical representations of regression equation. They provided a method to visualize the relationship between responses and experimental levels of each variable and the type of interactions between two test variables. The shapes of the contour plots, circular or elliptical, indicate whether the mutual interactions between the variables are significant or not. Circular contour plot indicates that the

**Table 2**
The coded experimental and predicted for RSM design using water as solvent.

| Run | Enzyme treated time (min, $X_1$) | Liquid–solid ratio ($X_2$) | Enzyme amount (%, $X_3$) | Enzyme treated temperature (°C, $X_4$) | Extraction yield (%) | |
|---|---|---|---|---|---|---|
| | | | | | Experimental | Predicted |
| 1 | 0 (60) | 0 (12) | 0 (0.30) | 0 (55) | 15.48 | 15.69 |
| 2 | 1 (90) | 0 (12) | −1 (0.20) | 0 (55) | 14.72 | 13.47 |
| 3 | 1 (90) | 0 (12) | 0 (0.30) | −1 (45) | 15.98 | 13.48 |
| 4 | 1 (90) | 0 (12) | 0 (0.30) | 1 (65) | 15.79 | 14.50 |
| 5 | −1 (30) | 1 (14) | 0 (0.30) | 0 (55) | 13.65 | 14.38 |
| 6 | 0 (60) | 0 (12) | 0 (0.30) | 0 (55) | 13.59 | 13.34 |
| 7 | −1 (30) | −1 (10) | 0 (0.30) | 0 (55) | 13.74 | 12.96 |
| 8 | 0 (60) | 1 (14) | 1 (0.40) | 0 (55) | 13.48 | 15.52 |
| 9 | 0 (60) | 1 (14) | 0 (0.30) | −1 (45) | 15.88 | 13.97 |
| 10 | 0 (60) | 0 (12) | 1 (0.40) | 1 (65) | 15.66 | 13.58 |
| 11 | 0 (60) | 0 (12) | 0 (0.30) | 0 (55) | 13.96 | 14.56 |
| 12 | 0 (60) | −1 (10) | 0 (0.30) | −1 (45) | 13.14 | 13.76 |
| 13 | 0 (60) | −1 (10) | −1 (0.20) | 0 (55) | 15.50 | 13.83 |
| 14 | 0 (60) | −1 (10) | 0 (0.30) | 1 (65) | 15.96 | 14.15 |
| 15 | 0 (60) | 1 (14) | 0 (0.30) | 1 (65) | 14.15 | 15.50 |
| 16 | −1 (30) | 0 (12) | −1 (0.20) | 0 (55) | 13.87 | 13.99 |
| 17 | 0 (60) | 1 (14) | −1 (0.20) | 0 (55) | 14.19 | 14.54 |
| 18 | 1 (90) | −1 (10) | 0 (0.30) | 0 (55) | 13.97 | 13.66 |
| 19 | −1 (30) | 0 (12) | 1 (0.40) | 0 (55) | 14.52 | 15.02 |
| 20 | 0 (60) | 0 (12) | 0 (0.30) | 0 (55) | 14.18 | 14.69 |
| 21 | 0 (60) | 0 (12) | −1 (0.20) | −1 (45) | 13.45 | 14.16 |
| 22 | −1 (30) | 0 (12) | 0 (0.30) | 1 (65) | 15.04 | 13.18 |
| 23 | 0 (60) | 0 (12) | 1 (0.40) | −1 (45) | 15.82 | 14.15 |
| 24 | 1 (90) | 1 (14) | 0 (0.30) | 0 (55) | 14.37 | 13.95 |
| 25 | 0 (60) | 0 (12) | −1 (0.20) | 1 (65) | 14.56 | 15.88 |
| 26 | −1 (30) | 0 (12) | 0 (0.30) | −1 (45) | 13.35 | 15.88 |
| 27 | 0 (60) | 0 (12) | 0 (0.30) | 0 (55) | 14.51 | 15.88 |
| 28 | 1 (90) | 0 (12) | −1 (0.20) | 0 (55) | 12.93 | 15.88 |
| 29 | 1 (90) | 0 (12) | 0 (0.30) | −1 (45) | 14.00 | 15.88 |

**Table 3**
ANOVA for the effects of enzyme treated time ($X_1$), enzyme amount ($X_2$), liquid–solid ratio ($X_3$) and enzyme treated time ($X_4$) on extraction yield of MFSCP with water as solvent using predicted polynomial models.

| Source | Sum of squares | Df | Mean square | *F*-value | *P*-value | Significant |
|---|---|---|---|---|---|---|
| Model | 24.63 | 14 | 1.76 | 594.59 | <0.0001 | *** |
| $X_1$ | 1.08 | 1 | 1.08 | 364.52 | <0.0001 | *** |
| $X_2$ | 1.05 | 1 | 1.05 | 354.72 | <0.0001 | *** |
| $X_3$ | 1.71 | 1 | 1.71 | 579.21 | <0.0001 | *** |
| $X_4$ | 0.43 | 1 | 0.43 | 146.76 | <0.0001 | *** |
| $X_1$ $X_2$ | 2.62 | 1 | 2.62 | 886.46 | <0.0001 | *** |
| $X_1$ $X_3$ | 0.08 | 1 | 0.08 | 25.48 | 0.0002 | ** |
| $X_1$ $X_4$ | 0.04 | 1 | 0.04 | 14.68 | 0.0018 | ** |
| $X_2$ $X_3$ | 0.84 | 1 | 0.84 | 282.65 | <0.0001 | *** |
| $X_2$ $X_4$ | 0.15 | 1 | 0.15 | 52.22 | <0.0001 | *** |
| $X_3$ $X_4$ | 3.23 | 1 | 3.23 | 1090.99 | <0.0001 | *** |
| $X_1^2$ | 3.59 | 1 | 3.59 | 1211.79 | <0.0001 | *** |
| $X_2^2$ | 4.74 | 1 | 4.74 | 1601.33 | <0.0001 | *** |
| $X_3^2$ | 2.85 | 1 | 2.85 | 962.99 | <0.0001 | *** |
| $X_4^2$ | 8.91 | 1 | 8.91 | 3012.72 | <0.0001 | *** |
| Residual | 0.04 | 14 | $2.96 \times 10^{-3}$ | | | |
| Lack of fit | 0.01 | 10 | $1.34 \times 10^{-3}$ | 0.19 | 0.96 | No significant |
| Pure error | 0.03 | 4 | $7.02 \times 10^{-3}$ | | | |
| Cor total | 24.67 | 28 | | | | |
| $R^2$ | 24.63 | 14 | 1.76 | 594.59 | | |
| Adj. $R^2$ | 1.08 | 1 | 1.08 | 364.52 | | |
| Pred. $R^2$ | 1.05 | 1 | 1.05 | 354.72 | | |
| Adequate precision | 1.71 | 1 | 1.71 | 579.21 | | |

** Significant at $P < 0.01$.
*** Significant at $P < 0.001$.

interactions between the corresponding variables are negligible, while elliptical contour plot indicates that the interactions between the corresponding variables are significant. In this study, the results of extraction yield of *CFMPS* affected by enzyme treated time, enzyme amount, liquid–solid ratio and enzyme treated temperature is presented in Fig. 1.

Fig. 1a, which give the extraction yield of *CFMPS* as a function of enzyme treated time and liquid–solid ratio at fixed enzyme amount (0.30%) and enzyme treated temperature (55 °C), indicated that the extraction yield increased rapidly with increase in enzyme treated time from 30 to 50 min, and increased with the increase of liquid–solid ratio from 10 to 11. However, the extraction yield decrease rapidly with the enzyme treated time increasing from 50 to 90 min and liquid–solid ratio from 11 to 14.

The extraction yield of *CFMPS* affected by different enzyme treated time and enzyme amount was shown in Fig. 1b, when the

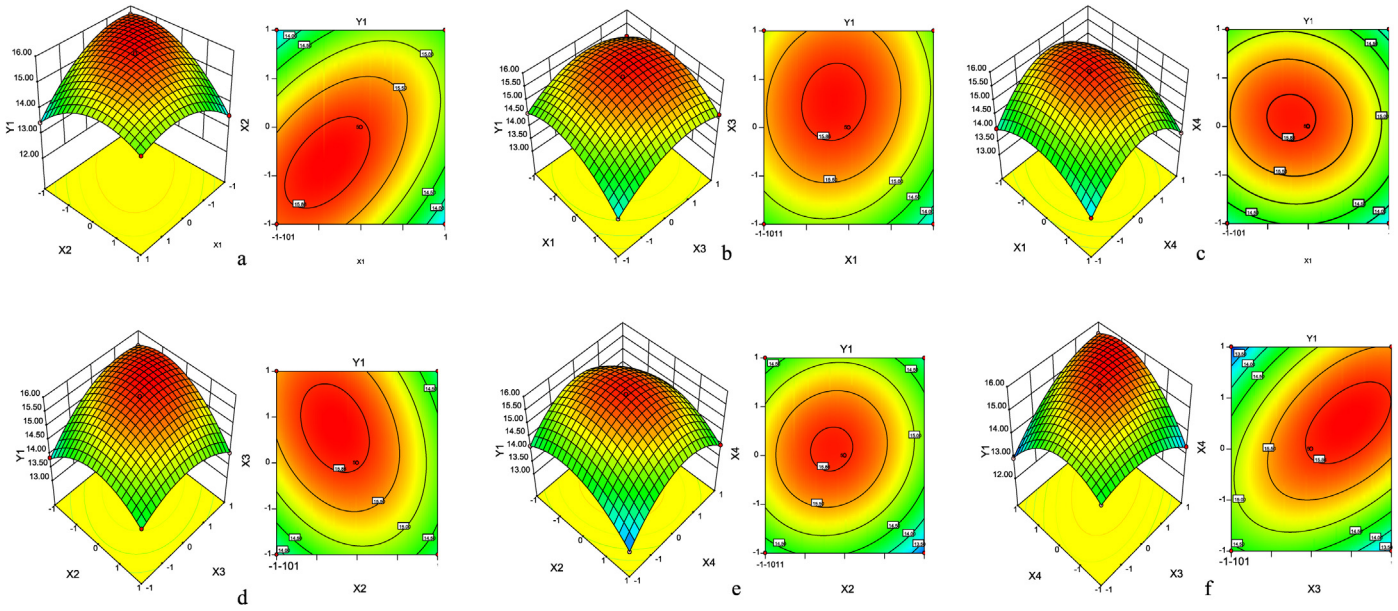


**Fig. 1.** Response surface (3D) and Contour plots showing the effect of enzyme treated time ($X_1$), enzyme amount ($X_2$), liquid–solid ratio ($X_3$) and enzyme treated time ($X_4$) on extraction yield of crude *MFPS*.

liquid solid ratio and enzyme treated temperature were fixed at 12 and 55 °C respectively. From the two figures, we can conclude that enzyme treated time and enzyme amount had a positive impact on the extraction yield of *CFMPS*. The extraction yield of *CFMPS* was found to increase slowly with increase of enzyme treated time from 30 to 55 min and decrease rapidly with increase of enzyme treated time from 55 to 90 min. The extraction yield of *CFMPS* increased with the enzyme amount from 0.20% to 0.33%, but beyond 0.35%, extraction yield of *CFMPS* reached the plateau region where the yield was maximized and did not further increase the yield. It can be seen that the maximum extraction yield of *CFMPS* can be achieved when enzyme amount and extraction time are around 0.35% and 55 min, respectively.

Fig. 1c shows the 3D response surface plot and the contour plot at varying enzyme treated time and enzyme extraction temperature at fixed liquid–solid ratio (0 level) and enzyme amount (0 level). It indicated that the maximum extraction yield of *CFMPS* can be achieved when enzyme treated time and enzyme extraction temperature at the threshold level of around 50 min and 55 °C, respectively.

Fig. 1d illustrates the 3D response surface plot and the contour plot at varying liquid–solid ratio and enzyme amount at fixed enzyme treated time (0 level) and enzyme treated temperature (0 level). The extraction yield of *CFMPS* decreased fleetly with the increasing liquid–solid ratio from 11.5 to 14 and reached the maximum value when the extraction temperature at the threshold level of 55 °C. So, extraction yield of *CFMPS* increased with decreasing of liquid–solid ratio and increasing enzyme amount.

In Fig. 1e, when the 3D response surface plot and the contour plot were developed for the extraction yield of *CFMPS* with varying liquid–solid ratio and enzyme treated temperature at fixed enzyme treated time (60 min) and enzyme amount (0.30%). The yield increased rapidly with the enzyme treated time. The maximum extraction yield of *CFMPS* achieved when liquid–solid ratio and enzyme extraction temperature at the threshold level of around 11.5 and 55 °C, respectively.

The 3D response surface plot and the contour plot based on the independent variable and enzyme amount and enzyme treated temperature was shown in Fig. 1f, while the other two independent variables, enzyme treated time and liquid–solid ratio were kept at 60 min and 12, respectively. An increase in the extraction yield of *CFMPS* could be significantly achieved with the increasing of enzyme amount. It was observed that the extraction yield of *CFMPS* increased with the enzyme treated temperature from 25 to 55 °C, and reached the maximum value at an extraction time around 58 °C, but beyond this time, extraction yield of *CFMPS* decreased.

### 3.7. Verification of predictive model, DPPH free radical scavenging ability, and ADH activity

Response surface optimization is more advantageous than the traditional single parameter optimization in that it saves time, space and raw material. In order to validate the adequacy of the model equations, verification experiment was carried out under the optimal conditions: enzyme amount 0.30%, enzyme treated time 38 min, enzyme treated temperature 58 °C and liquid–solid ratio 11.0. Good agreement exist between the values predicted using model equations and the experimental values at the points of interest. To ensure the predicted result was not biased toward the practical value, experimental rechecking was performed using this deduced optimal condition. This set of conditions was determined to be optimal by the RSM optimization approach and was also used to validate experimentally and predict the values of the response using the model equation. A mean value of $16.16 \pm 0.14\%$ ($n = 5$), obtained from real experiments, demonstrated the validation of *RSM* model. The validation result revealed that there was no significant difference between experimental and predicted values, suggesting that the response model was adequate for reflecting the expected optimization (Table 4). This result of analysis indicated that the experimental values were good agreement with the predicted ones, and also suggested that the model of Eq. (6) is satisfactory and accurate.

Furthermore, none enzyme treated process conditions were compared with the above optimized ones, as seen in Table 4, a mean value of extraction yield $10.15 \pm 0.05\%$ ($n = 3$) obtained from the none enzyme treated samples, extraction yield of enzyme treated condition increased 161% compared with the none enzyme treated ones.

The scavenging effects of *F. Mori* extracts on DPPH radical were measured and shown in Fig. 2a. The scavenging effects of

**Table 4**
Result of model validation experiments.

| No. | Optimum conditions | | | | Extraction yield of *MFPS* (%) | |
|---|---|---|---|---|---|---|
| | Enzyme treated time (min) | Enzyme amount (%) | Liquid–solid radio | Enzyme treated temperature (°C) | Experimental | Predicted |
| 1 | 38.0 | 0.3 | 11.0 | 58.0 | 16.20 | 16.17 |
| 2 | 38.0 | 0.3 | 11.0 | 58.0 | 16.08 | 16.17 |
| 3 | 38.0 | 0.3 | 11.0 | 58.0 | 16.12 | 16.17 |
| 4 | 38.0 | 0.3 | 11.0 | 58.0 | 16.15 | 16.17 |
| 5 | 38.0 | 0.3 | 11.0 | 58.0 | 16.23 | 16.17 |
| Average | | | | | 16.16 | 16.17 |
| 6 | 0 | 0.3 | 11.0 | 58.0 | 10.21 | |
| 7 | 0 | 0.3 | 11.0 | 58.0 | 10.13 | |
| 8 | 0 | 0.3 | 11.0 | 58.0 | 10.11 | |
| Average | | | | | 10.15 | |

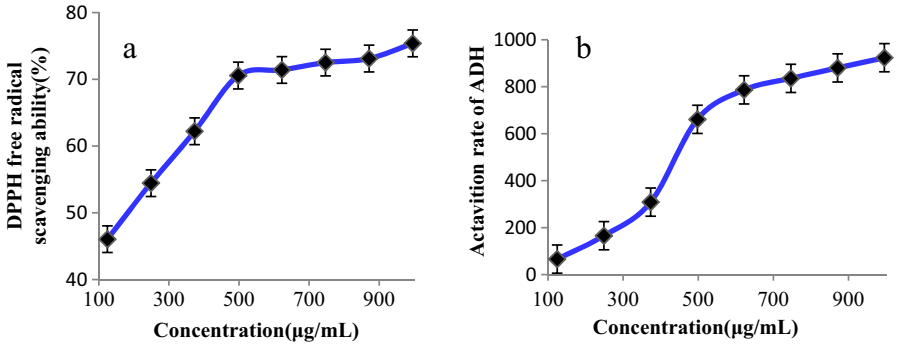


**Fig. 2.** Effect of *MFPS* on the scavenging rate of *DPPH* radical (a) and activities of *ADH* (b).

*CFMPS* obtained by the extraction conditions of glucose oxidase assisted extraction increased significantly with the concentrations (125–995 μg mL$^{-1}$) increasing and were stronger than that of extracts obtained using the method before optimized by *RSM*.

The activation of *CFMPS* on *ADH* were measured and shown in Fig. 2b. The development of *ADH* activity could be affected by *CFMPS* obtained from the extraction conditions of glucose oxidase extraction significantly increased with the concentrations (125–995 μg mL$^{-1}$) increasing and were stronger than that of extracts acquired using the method before optimized by *RSM*.

## 4. Conclusions

In the present study, four single enzyme extraction methods, three compound enzymes extraction methods and none enzyme extraction methods were screened for the pre-extraction treatment of *F. Mori*, and the extracts exhibited different yields, levels of scavenging effects on *DPPH* free radical and rate activation of *ADH*. Glucose oxidase was found to be the most effective one for improving *FMPS* yield among the tested enzymes. All extracts obtained from *F. Mori* exhibited different *DPPH* free radical scavenging effects and rate activation of *ADH*, and the glucose oxidase extract had the highest scavenging effects on *DPPH* and higher activation rate of *ADH*. In the case of water as solvent, optimal extraction conditions for glucose oxidase assisted extract of *CFMPS* were obtained as follows conditions: enzyme amount 0.30%, enzyme treated time 38 min, enzyme treated temperature 58 °C and liquid–solid radio 11.0. Under this condition, the mean experimental value of extraction yield (16.16 ± 0.14%) was achieved, which corresponds well with the predicted values and increased more than 160% compared with the none enzyme treated ones. The antioxidant and ADH in vitro of CFMPS obtained by the extraction conditions of glucose oxidase extraction increased significantly with the concentrations (125–995 μg mL$^{-1}$) increasing and were stronger than that of extracts obtained using the extraction conditions before optimized by *RSM*.

## Acknowledgements

The authors gratefully acknowledge the financial support of the present work by the National Sci-Tech Support Plan of China (2011BAC09B01), and Guizhou Province Science and Technology Plan Projects (Nos. 2012401-4, 2013-5005, KY-2012-005, 2013-2068 and ZY-2011-3013).

## References

Abbasi, A. M., Khan, M. A., Khan, N., & Shah, M. H. (2013). Ethnobotanical survey of medicinally important wild edible fruits species used by tribal communities of Lesser Himalayas-Pakistan. *Journal of Ethnopharmacology*, *148*(2), 528–536.

Cohen, G. H., & Johnstone, D. B. (1964). Extracellular polysaccharides of azotobacter vinelandii. *Journal of Bacteriology*, *88*, 329–338.

Edwards, A., Mur, L. A., Girdwood, S. E., Anesio, A. M., Stibal, M., Rassner, S. M., et al. (2014). Coupled cryoconite ecosystem structure-function relationships are revealed by comparing bacterial communities in alpine and Arctic glaciers. *FEMS Microbiology Ecology*, http://dx.doi.org/10.1111/1574-6941.12283

Francisco, E. C., Franco, T. T., Wagner, R., & Jacob-Lopes, E. (2014). Assessment of different carbohydrates as exogenous carbon source in cultivation of cyanobacteria. *Bioprocess and Biosystems Engineering*, http://dx.doi.org/10.1007/s00449-013-1121-1

Hosseinpour, M., Vossoughi, M., & Alemzadeh, I. (2014). An efficient approach to cathode operational parameters optimization for microbial fuel cell using response surface methodology. *Journal of Environmental Health Science and Engineering*, *12*(1), 33.

Jiang, D.-Q., Guo, Y., Xu, D.-H., Huang, Y.-S., Yuan, K., & Lv, Z.-Q. (2013). Antioxidant and anti-fatigue effects of anthocyanins of mulberry juice purification (MJP) and mulberry marc purification (MMP) from different varieties mulberry fruit in China. *Food and Chemical Toxicology*, *59*(0), 1–7.

Kim, H. G., Ju, M. S., Shim, J. S., Kim, M. C., Lee, S. H., Huh, Y., et al. (2010). Mulberry fruit protects dopaminergic neurons in toxin-induced Parkinson's disease models. *British Journal of Nutrition*, *104*(1), 8–16.

Kim, J. H., Chung, H. S., Kang, M., Kim, Y., Kim, B. S., Kim, Y. S., et al. (2011). Anti-diabetic effect of standardized herbal formula PM021 consisting of Mori Folium and Aurantii Fructus on type II diabetic Otsuka Long-Evans Tokushima Fatty (OLETF) rats. *Diabetes Research and Clinical Practice*, *93*(2), 198–204.

Kumar, S. P. (2013). Optimization of lipid enriched biomass production from oleaginous fungus using response surface methodology. *Indian Journal of Experimental Biology*, *51*(11), 979–983.

Lee, S.-L., Wang, M.-F., Lee, A.-I., & Yin, S.-J. (2003). The metabolic role of human ADH3 functioning as ethanol dehydrogenase. *FEBS Letters*, *544*(1–3), 143–147.

Liu, C.-J., & Lin, J.-Y. (2012). Anti-inflammatory and anti-apoptotic effects of strawberry and mulberry fruit polysaccharides on lipopolysaccharide-stimulated macrophages through modulating pro-/anti-inflammatory cytokines secretion and Bcl-2/Bak protein ratio. *Food and Chemical Toxicology*, *50*(9), 3032–3039.

Nomura, T., & Fukai, T. (1981). Constituents of the cultivated mulberry tree. *Planta Med*, *42*(1), 79–88.

Pauchet, Y., Saski, C. A., Feltus, F. A., Luyten, I., Quesneville, H., & Heckel, D. G. (2014). Studying the organization of genes encoding plant cell wall degrading enzymes in Chrysomela tremula provides insights into a leaf beetle genome. *Insect Molecular Biology*, *23*(3), 286–300.

Saha, A. K., & Brewer, C. F. (1994). Determination of the concentrations of oligosaccharides, complex type carbohydrates, and glycoproteins using the phenol–sulfuric acid method. *Carbohydrate Research*, *254*, 157–167.

Tang, C.-C., Huang, H.-P., Lee, Y.-J., Tang, Y.-H., & Wang, C.-J. (2013). Hepatoprotective effect of mulberry water extracts on ethanol-induced liver injury via anti-inflammation and inhibition of lipogenesis in C57BL/6J mice. *Food and Chemical Toxicology*, *62*, 786–796.

Tian, C.-Y., Bo, H.-M., & Li, J.-A. (2011). Influence of Mori Fructus Polysaccharide on Blood Glucose and Serum Lipoprotein in Rats with Experimental Type 2 Diabetes. *Chinese Journal of Experimental Traditional Medical Formulae*, *17*(10), 158–160.

Vallee, B. L., & Hoch, F. L. (1955). Zinc, a component of yeast alcohol dehydrogenase. *Proceedings of the National Academy of Sciences of the United States of America*, *41*(6), 327–338.

Wang, C. C., Chang, S. C., Inbaraj, B. S., & Chen, B. H. (2010). Isolation of carotenoids, flavonoids and polysaccharides from *Lycium barbarum* L. and evaluation of antioxidant activity. *Food Chemistry*, *120*(1), 184–192.

Yang, B., Zhao, M., Shi, J., Yang, N., & Jiang, Y. (2008). Effect of ultrasonic treatment on the recovery and DPPH radical scavenging activity of polysaccharides from longan fruit pericarp. *Food Chemistry*, *106*(2), 685–690.

Yoshinaga, N., Aboshi, T., Ishikawa, C., Fukui, M., Shimoda, M., Nishida, R., et al. (2007). Fatty acid amides, previously identified in caterpillars, found in the cricket Teleogryllus taiwanemma and fruit fly Drosophila melanogaster larvae. *Journal of Chemical Ecology*, *33*(7), 1376–1381.