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Optimization of enzymatic pretreatment for *n*-hexane extraction of oil from *Silybum marianum* seeds using response surface methodology

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ABSTRACT

An investigation on enzymatic pretreatment for *n*-hexane extraction of oil from the *Silybum marianum* seeds was conducted. The optimum combination of extraction parameters was obtained with the response surface methodology (RSM) at a four-variable and five-level central composite design (CCD). The optimum parameters of enzymatic pretreatment were as follows: enzyme concentration of 2.0% (w/w), temperature of 42.8 °C, reaction time of 5.6 h, and pH of 4.8. After enzymatic pretreatment, the oil was extracted by *n*-hexane for 1.5 h, and the oil yield on a dry basis was 45.70%, which well matched with the predicted value (45.86%). The results of the effects of the enzymatic pretreatment for *n*-hexane extraction of oil from the aspects of oil yield, microstructure and the fatty acid compositions showed that the enzymatic pretreatment had not affected on the fatty acid compositions, but could cause structure breakage of the *S. marianum* seeds and accelerate releasing extra oil, which increased the oil yield by 10.46% compared with *n*-hexane extraction for 1.5 h without enzymatic pretreatment, and confirmed the efficacy of enzymatic pretreatment for *n*-hexane extraction of oil from the *S. marianum* seeds.

Keywords: *Silybum marianum*; Seed oil; Enzymatic pretreatment; Response surface methodology; Fatty acid composition; Scanning electron microscopy

1. Introduction

Silybum marianum (family: Compositae) is an annual or biennial plant, native to the Mediterranean area, and now has spread to other warm and dry regions. In China, it mainly distributes in Qinghai, Shanxi, Hubei, Jiangsu and Guangdong provinces. Various preparations of the plant, especially the fruits, have been used medicinally to treat liver disorders for over 2000 years (Flora et al., 1998). Besides the cytoprotective activity, there is a growing interest in its anticancer as well as in its chemopreventive, hypocholesterolemic, cardioprotective, neuroactive and neuroprotective activities (Sobolova et al., 2006). Silymarin is the pharmacological active principle of the fruits and composed of an isomeric mixture of the flavonolignans silychristin, silydianin, the diastereoisomers silybin and isosilybin (Morazzoni and Bombardelli, 1995).

The *S. marianum* contains a relatively high amount of oil, which makes one step extraction of silymarin from the fruit impossible. The oil is by-product of silymarin industrial production and has to be removed from seeds prior to the silymarin extraction. The oil contains essential phospholipids, a relatively high content of vitamin E (Hadolin et al., 2001), and a great quantity of the unsaturated fatty acids such as linoleic acid (C18:2) and oleic acid (C18:1) (Yin et al., 1998). Therefore, the *S. marianum* seeds would be as a novel source of the plant oils.

The oil from the plant seeds has been traditionally recovered by hydraulic pressure and solvent extraction (Dibert et al., 1989; Mani et al., 2007). The oil yields of the process

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are significantly increased by mechanical or thermal condition (Pradhan et al., 2009; Zabaras et al., 2002). Limitations of these processes include the requirements of special instruments and energy consummation. Enzymatic pretreatment has recently shown to be another alternative method, which opens up cell walls through biodegradation and releases oil from plants, thus serves the same purpose as the conventional pretreatment. In addition, it breaks up the lipoprotein and lipopolysaccharide molecules into simpler molecules and releases extra oil from the plant (Dominguez et al., 1995a,b; Jiang et al., 2010; Shankar et al., 1997). However, enzymatic pretreatment is influenced by several factors such as enzyme concentration, temperature, time and pH, which cooperatively affect the enzyme activity. In most previous studies, the process conditions have been merely optimized by conducting one factor-at-a-time experiments (Passos et al., 2009) which do not reflect actual changes in the environment as they ignore interactions among factors simultaneously. Therefore, these factors may be collectively studied to validate the optimal extraction conditions.

The response surface methodology (RSM) has been demonstrated to be a powerful tool for determining the factors and their interactions, which allow process optimization to be conducted effectively (Liu et al., 2009). The method is the preferred experimental design for fitting polynomial model to analyze the response surface of multi-factor combinations, and a faster and more economical method for gathering research results than classic one-variable-at-a-time or full-factors experimentation. However, to the best of our knowledge, there is little information on enzymatic pretreatment for oil extraction from the *S. marianum* seeds. In order to utilize the oil and enhance the oil yield of *S. marianum* seeds in food processing industry, the present study was to optimize the process parameters (enzyme concentration, temperature, reaction time and pH) of enzymatic pretreatment for *n*-hexane extraction of oil from the *S. marianum* seeds with RSM, and to evaluate the effects of the enzymatic pretreatment in the aspects of oil yield, microstructure and the fatty acid compositions of *S. marianum* seeds.

2. Materials and methods

2.1. Materials and chemicals

The *S. marianum* fruits and the oil produced via hydraulic pressure (HP) were obtained from Zhongxing Pharmaceutical Co., Ltd (Zhenjiang, Jiangsu, China). The fruits were identified by Chen Li (Chief Pharmacist, Food and Drug Administration Bureau of Zhenjiang). The seeds were obtained from the *S. marianum* fruits, husked and air-dried at room temperature and crashed to average particle size of 300 μm. The commercial enzymes (including cellulase, xylanase, pectinase and protease) employed for the optimization studies were purchased from Hemei Biology Co., Ltd (Jingning Shandong, China). Cellulase (activity of 10,000 LEU/g), xylanase (activity of 10,000 LEU/g), pectinase (activity of 10,000 LEU/g) and protease (activity of 100,000 LEU/g) were produced from *Aspergillus niger*, *Thermomyces lanuginosus*, *Aspergillus aculeatus* and *Bacillus amyloliqefaciens*, respectively. All solvents and chemicals were analytical grade and obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

2.2. Enzymatic pretreatment

Prior to the *n*-hexane extraction of *S. marianum* seed oil, an enzymatic pretreatment was performed according to the method described by Passos et al. (2009) with some modifications. The commercial enzymes were dispersed in deionized water to obtain different concentrations of the enzymatic suspension. The ratio of enzymatic suspension and seeds was kept at 4.0 mL/g (dry basis) during all experiments based on the earlier studies (Rosenthal et al., 1996). 10 g seed powders were treated with enzymatic cocktails of cellulase, xylanase, pectinase and protease (at the proportion of 2:1:1:2). The pH was fixed with a buffer solution of citric acid and disodium hydrogen phosphate. The reaction proceeded isothermally, under continuous stirring at 200 rpm, and stopped by freezing the suspension with liquid nitrogen. The resultant samples were finally lyophilized.

2.3. Oil extraction

The oil extraction was carried out with 150 mL of *n*-hexane in a Soxhlet apparatus for 1.5 h. The solution were treated by anhydrous sodium sulphate for 12 h, filtered by a G1 sintered glass filter, and then evaporated in a rotary evaporator at 45 °C. The oil yields were expressed as the mass of oil extracted from 100 g dried *S. marianum* seeds.

2.4. Experimental design and statistical analysis

A four-variable and five-level central composite design (CCD) was performed in the present study to optimize the process parameters of enzymatic pretreatment for *n*-hexane extraction of oil from *S. marianum* seeds. The four independent variables were enzyme concentration (X_1), temperature (X_2), reaction time (X_3) and pH (X_4). A set of 32 experiments with four variables were required (Table 1). The average oil yield (%) was taken as the response (Y). For statistical calculations, the relation between the coded values and actual values were described by Eq. (1):

$$x_i = \frac{X_i - X_0}{\Delta X} \quad (1)$$

where x_i is the coded value of the independent variable, X_i is the actual value of the variable, X_0 is the actual value at the center point, and ΔX is the step change in the variable X_i. The uncoded and coded levels of the independent variables used in CCD are listed in Table 2.

The levels of the independent parameters were based on preliminary experimental results. Triplicate experiments were carried out at all designed points except at the central point (0, 0, 0), where eight replications were performed to allow the estimations of "pure error". All experiments were carried out in randomized order to minimize the effect of unexplained variability in the observed responses due to extraneous factors.

The quadratic equation for the variables shown in Eq. (2):

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_i X_i^2 + \sum \sum \beta_{ij} X_i X_j \quad (2)$$

where Y = predicted response, β_0 = a constant, β_i = linear coefficient, β_{ii} = squared coefficient, and β_{ij} = interaction coefficient. X_i and X_j are the independent variables. Eq. (2) was used to build surfaces for variables. A software Design-Expert 7.1.3