Optimization of Extraction key Process for Erinacine from Hericium erinaceus

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Abstract-In order to improve the yield of erinacine from H. erinaceus, with pretreatment of enzymatic and acid hydrolysis, then combined with the method of industrial alcohol reflux extraction, significantly the extraction yield of erinacine was improved from 2.4% to 3.2%. In order to optimize extraction process parameters for erinacine from H. erinaceus, the content of erinacine in H. erinaceus as the raw material was measured to evaluate the effects of extraction liquid/material ratio, extraction ethanol/water ratio, extraction temperature and extraction time by single factor experiments and response surface methodology (RMS). Results indicated that the optimal extraction conditions for erinacine from H. erinaceus were extraction liquid/material ratio of 32ml/g, ethanol/water ratio of 65%, extraction temperature of 62 ℃ and extraction time of 30min.Under the condition of the correction, the actual measured extraction yield of erinacine was 3.28%.

Keywords- erinacine; enzymatic and acid hydrolysis; yield; response surface.

I. Introduction

H. erinaceus in taxonomy attached fungus world, Basidiomycota, basidiomycetes, Hericium genus. Previous studies reported that the fungal mycelia of H. erinaceus contains various bioactive constituents such as polysaccharides(1), proteins, lectin(2), hericenones(3), erinacines(4, 5), sterol, fatty acid and esters(6). H. erinaceus has beneficial effects, including anti-cancer activity(7), antibacterial activity(8, 9), anti-inflammatory activity(10, 11), antioxidant properties(12) and stimulating the synthesis of nerve growth factor(13).

In the previous papers, erinacine which was extracted from H. erinaceus exhibited potent stimulating activity to NGF-synthesus(14, 15), antibacterial activity and antioxidant properties. Stimulators of NGF-synthesis had been expected to be used as drugs for degenerative neuronal disorders such as Alzheimer's disease and for peripheral nerve regeneration(4, 5). Since the yield of erinacine was very low, as reported in the literature using solvent reflux extraction, microwave extraction, ultrasonic extraction and supercritical fluid extraction rate was less than 2.4% and the extraction costs were high, so the further extraction and exploitation of erinacine became the bottleneck(16).

In order to improve the yield of erinacine from H. erinaceus, with pretreatment of enzymatic and acid hydrolysis, then combined with the method of industrial alcohol reflux extraction, significantly the extraction yield of erinacine improve from 2.4% to 3.2%.Based on single factor experiments and response surface methodology (RSM), the extraction process parameters were optimized for erinacine extraction provides a good production prospects.

II. Materials and methods

A. Materials

Fungal mycelia of H. erinaceus powder(Beijing f u er kang biotechnology research institute), 95%indust rial alcohol, hydrochloric acid, acetic acid, sulfuric ac

id, perchloric acid,absolute ethyl alcohol, vanillin, petr oleum ether, ethyl acetate, cellulose enzyme, wild sta ndard scutellarin.

B. Instrument

UV-7504ultraviolet and visible spectrophotometert her, mostat water bath, AL-104electronic scales, RE-2 000Arotary evaporators.

C. Experimental method

1) Technological process

The fungal mycelia of H. erinaceus powder produced by drying→Enzymatic hydrolysis with cellulase→Destroy the enzyme activity→evaporated to dryness→Industrial alcohol solvent reflux extraction→Filtration→Uv-vis spectrophotometer measure of absorbance values→The optimal technological conditions were obtained.

2) Test method

a) Constant temperature solvent extraction

The dry fungal mycelia of H. erinaceus powder was accurately weighed 10 g (accurate to 0.0001 g) by AL-104 electronic scales. The fungal mycelia of H. erinaceus powder was circularly extracted with 100ml of 95% ethanol solvent in a 500 ml round bottom flask.

b) Pretreatment of enzymatic hydrolysis solvent extraction

The dry fungal mycelia of H. erinaceus powder was accurately weighed 10 g (accurate to 0.0001 g) by AL-104 electronic scales. The dry fungal mycelia of H. erinaceus powder was put in 250 ml beaker containing 100 ml of water and 0.1 g of cellulose enzyme. The pH value was adjusted to 4.5 by hydrochloric acid. After enzymatic hydrolysis 90 min of 50℃ and inactivated boil for 10 minutes, the combined extracts were then concentrated on rotary evaporator at 50℃ to remove water to dryness. The fungal mycelia of H. erinaceus powder was circularly extracted with 100ml of 95% ethanol solvent in a 500 ml round bottom flask.

c) Pretreatment of acid hydrolysis solvent extraction

The dry fungal mycelia of H. erinaceus powder was accurately weighed 10 g (accurate to 0.0001 g) by AL-104 electronic scales. The dry fungal mycelia of H. erinaceus powder was put in 250 ml beaker containing 100 ml of water and 0.1 g of cellulose enzyme. The pH value was adjusted to 2 by hydrochloric acid. After acid hydrolysis 24h, he combined extracts were then concentrated on rotary evaporator at 50℃to remove water to dryness. The fungal mycelia of H. erinaceus powder was circularly extracted with 100ml of 95% ethanol solvent in a 500 ml round bottom flask.

d) Pretreatment of both enzymatic hydrolysis and acid hydrolysis solvent extraction

The dry fungal mycelia of H. erinaceus powder was accurately weighed 10 g (accurate to 0.0001 g) by AL-104 electronic scales. The dry fungal mycelia of H. erinaceus powder was put in 250 ml beaker containing 100 ml of water and 0.1 g of cellulose enzyme. After enzymatic hydrolysis 90 min of 50℃ and inactivated boil for 10 minutes,the pH value was adjusted to 2 by hydrochloric acid. After acid hydrolysis 24h, he combined extracts were then concentrated on rotary evaporator at 50℃to remove water to dryness. The fungal mycelia of H. erinaceus powder was circularly extracted with 100ml of 95% ethanol solvent in a 500 ml round bottom.

D. Qualitative appraisal of erinacine

Erinacine existed in alcohol extract was measured by thin layer chromatographic analysis of developing solvent of ethyl acetate and petroleum ether system as the color of terpenoids general indicators respective were ethanol and vanillin sulfuric acid sulfate ethanol.

E. Maximum absorption maxima and calibration plot

2 mL diluted extract in quartz colorimetric utensil was scanned between 200 ~ 400 nm wavelength，then the operation was repeated for three times. Its maximum absorption peak was discovered at about 210 nm（UV), so 210 nm（UV) was selected for detective wavelength.

Wild standard scutellarin which had strong absorption peak around 210 nm（UV）was accurately weighed 0.02g(accurate to 0.0001 g) . And the wild standard scutellarin was dissolved in 25mL absolute ethyl alcohol for 0.8mg/mL standardized solution. 0.2、0.4、0.6、0.8、 1.0mL of 0.8mg/mL standardized solution was accurately transferred into the 50 mL volumetric flask respectively and was added up to 50mL. The different

concentration standardized solution was shook for uniformity and the OD value was measured in 210nm （UV） respectively. Calibration plot was drew with the OD value Y as the ordinate and the concentration X as abscissa.regression equation ： Y=79.872X-0.0369 ， R^2=0.9981.Extraction yield linear range was 0.82% ~ 4.07% and the content of the sample was calculated by the calibration plot.

The yield of erinacine from H. erinaceus was measured by ultraviolet spectroscopy. H. erinaceus extraction rate/%=c*V*n/m*100% (1)

Formula：c was the concentration of erinacine，mg/mL；v wastotal volume,mL；n was the dilution ratio；m was the quality of H erinaceum powder, mg.

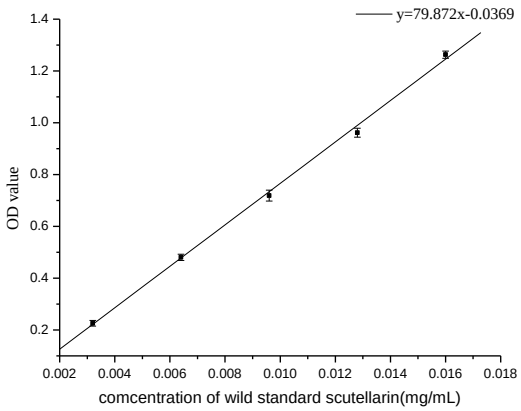

Figure 1. Calibration plot

III. Discussion and Results

A. Discussion

1) Different pretreatment on the effect of erinacine extraction yield

The erinacine extraction yield(Table 1) of four different pretreatment was compared. Then result showed that pretreatment of both enzymatic hydrolysis and acid hydrolysis solvent extraction can make the the yield of erinacine to improve significantly.

TABLE I.COMPARISION OF THE YEILD WITH DIFFERENT PRETREATMENT

	OD	Extraction yield/%
Constant temperature solvent extraction	0.536	1.793
Pretreatment of enzymatic hydrolysis solvent extraction	0.787	2.579
Pretreatment of acid hydrolysis solvent extraction	0.854	2.789
Pretreatment of both enzymatic hydrolysis and acid hydrolysis solvent extraction	0.983	3.193

2) Single factor experiment

a）The influence of extraction liquid/material ratio on erinacine extraction

H.erinaceum powder was accurately weighed 10 g(accurate to 0.0001 g) . It was extracted by diferent extraction liquid/material ratio（10:1,20:1,30:1,40:1,50:1）and other conditions about reflux extraction were ethanol/water ratio 95%, extraction temperature 60℃, extraction time 30min.

Figure 2 show that with the increase of liquid/material ratio the erinacine extraction yield was increased, but when the liquid/material ratio increased more than 30:1 the erinacine extraction yield tended to be stable. Maybe the erinacine had been extracted completely when the liquid/material ratio achieved 30:1. As the factors of the yield of erinacine, dosage of solvent and energy loss were considered comprehensively, the optimization extraction liquid/material ratio of the solvent refluxextraction was about 30:1.

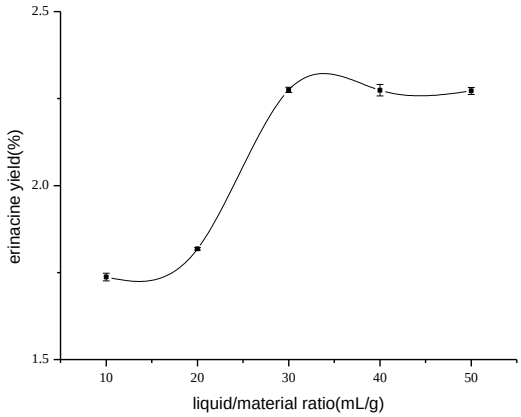

Figure 2. The influence of extraction liquid/material ratio on erinacine extraction

b) The influence of ethanol/water ratio on erinacine extraction

H.erinaceum powder was accurately weighed 10 g. It was extracted by diferent extraction ethanol/water ratio （55%, 65%, 75%, 85%, 95%）and other conditions about reflux extraction was extraction liquid/material ratio 30:1, extraction temperature 60℃, extraction time 30min.

Figure 3 show that with the increase of ethanol/water ratio the erinacine extraction yield was increased, but when the ethanol/water ratio increased more than 65% the erinacine extraction yield tended to decline. Maybe other substances of freely soluble in organic solvent were extracted which influenced the extraction yield of erinacine. So the optimization extraction ethanol/water ratio of the solvent reflux extraction was about 65%. Figure 3 also show that the influence of ethanol/water ratio change was not very great significant on erinacine extraction.

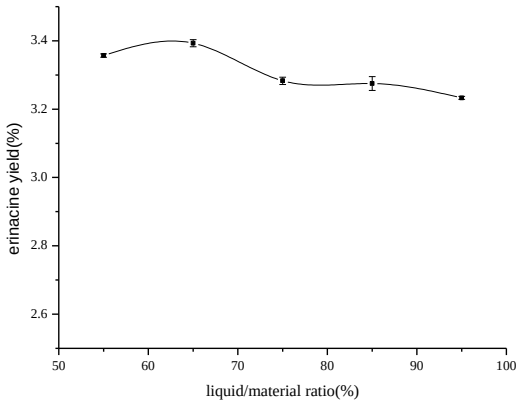

Figure 3. The influence of extraction ethanol/water ratio on erinacine extraction

c) The influence of extraction temperature on erinacine extraction

H.erinaceum powder was accurately weighed 10 g(accurate to 0.0001 g) . It was extracted by diferent extraction temperature（50℃, 60℃, 70℃, 80℃, 90℃） and other conditions about reflux extraction was extraction liquid/material ratio 30:1, extraction ethanol/water ratio 65%, extraction time 30min.

Figure 4 show that with the increase of extraction temperature the erinacine extraction yield was increased, but when the extraction temperature increased more than 70℃ the erinacine extraction yield tended to decline. The structure and biological activity of erinacine may be destroyed by the high temperature. So the optimization extraction temperature of the solvent reflux extraction was about 70℃.

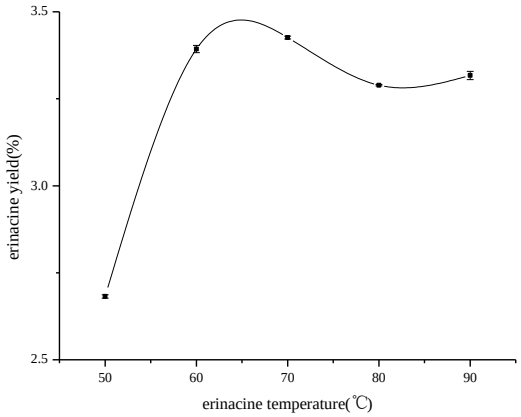

Figure 4. The influence of extraction temperature on erinacine extraction

d) The influence of extraction time on erinacine extraction

H.erinaceum powder was accurately weighed 10 g(accurate to 0.0001 g) . It was extracted by diferent extraction time（10min, 20min, 30min, 40min, 50min）and other conditions about reflux extraction was extraction

liquid/material ratio 30:1, extraction ethanol/water ratio 65%, extraction temperature 70℃.

Figure 5 show that with the increase of extraction time the erinacine extraction yield was increased, but when the extraction time increased more than 30min, the erinacine extraction yield tended to decline. The increase of time influenced the erinacine stability made erinacine extraction yield tended to decline after 30 min. So the optimization extraction time of the solvent reflux extraction was about 65%.

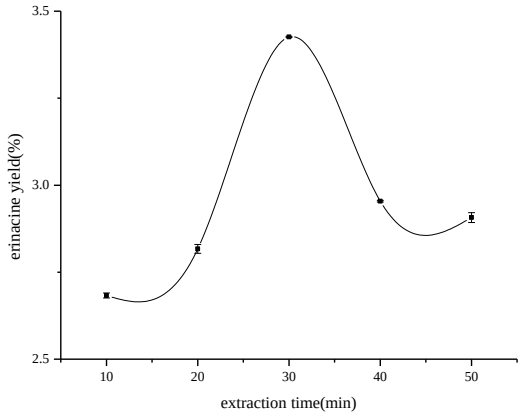

Figure 5. The influence of extraction time on erinacine extraction

3). Response surface methodology

According to the results of Single factor experiments (Fig. 2～5),three major influence factors (liquid/material ratio, extraction temperature, extraction time) were selected for next experiments because the influence of ethanol/water ratio change was not very great significant on erinacine yeild. On the basis of the single factor experiment results, the ranges of each factor were confirmed, and then a three variables (X_1, liquid/material ratio; X_2, extraction temperature; X_3, extraction time), three levels Box–Behnken design (DesignExpert software, Trial Version 8.0.6) was used to evaluate the best condition parameters for the extraction of erinacine. For statistical calculation, the above three values were coded as the following equation:

$X_1=(X_1-30)/10$, $X_2=(X_2-60)/10$, $X_3=(X_3-30)/10$. (2)

The experimental runs for Box–Behnken design were shown in Table 2. Each experimental run was per-formed in triplicate and the averages of the yield of erinacine were taken as response.

TABLE II. INDEPRNDENT VARIABLES AND THEIR LEVELS IN BOX–BEHNKEN DESIGN.

Independent variables	Symbol	Level		
		-1	0	1
Liquid/material ratio / mL/g	X_1	20	30	40
Extraction temperature / ℃	X_2	50	60	70
Extraction time / min	X_3	20	30	40

TABLE III. BOX–BEHNKEN EXPERIMENTAL DESIGN AND THE RESULTS FOR EXTRACTION YIELD OF ERINACINE(n = 3).

Run	Coded variable levels			Sample quality /g	Yield of erinacine /%
	X_1	X_2	X_3		
1	-1	-1	0	10.0003	2.828
2	1	-1	0	10.0010	2.770
3	-1	1	0	10.0011	2.916
4	1	1	0	10.0012	3.162
5	-1	0	-1	10.0006	2.812
6	1	0	-1	10.0012	2.893
7	-1	0	1	10.0021	2.836
8	1	0	1	10.0011	2.943
9	0	-1	-1	10.0004	2.790
10	0	1	-1	10.0013	2.837
11	0	-1	1	10.0014	2.746
12	0	1	1	10.0002	2.954
13	0	0	0	10.0005	3.355
14	0	0	0	10.0007	3.396
15	0	0	0	10.001	3.353
16	0	0	0	10.0006	3.385
17	0	0	0	10.0007	3.388

As shown in Table 3, each experiment in the design was performed and the experimental data were obtained. The data were analyzed by multiple regression analysis using the Design Expert software to get the following polynomial equation:

$$Y=3.38+0.047*X_1+0.092*X_2+0.018*X_3+0.076*X_1*X_2+6.500E\text{-}003*X_1*X_3+0.040*X_2*X_3-0.21*X_1^2-0.25*X_2^2-0.30*X_3^2 \quad (3)$$

TABLEIV. ANALYSIS OF VARIANCE OF THE EXPERIMENTAL RESULTS OF THE BOX–BEHNKEN DESIGN.

Variables	SS	DF	MS	F-value	P-value Prob > F
Model	1.02	9	0.011	100.20	< 0.0001
X_1	0.018	1	0.018	15.62	0.0055
X_2	0.068	1	0.068	59.67	0.0001
X_3	2.701E-003	1	7.503E-003	2.39	0.1663
X_1^2	0.018	1	0.18	161.87	< 0.0001
X_2^2	0.26	1	0.26	228.52	< 0.0001
X_3^2	0.37	1	0.37	325.61	< 0.0001
X_1X_2	0.023	1	0.023	20.42	0.0027
X_1X_3	1.690E-004	1	1.690E-004	0.15	0.7106

X_2X_3	6.480E-003	1	6.480E-003	5.73	0.0480
Residual	7.921E-003	7	1.132E-003		
Lack of fit	6.328E-003	3	2.109E-003	5.30	0.0706
pure error	1.5932E-003	4	3.9833E-004		
Cor Total	1.03	16			

The F-value and p-value were used to measure the significance of the coefficients of the model and results were shown in Table 4. 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. It also can be seen t hat the variables with the significant effects on the yield of erinacine were the linear terms (X_1, X_2), the quadratic terms (X_1^2, X_2^2 and X_3^2) and the interaction between X_1 and X_3, X_2 and X_3. the determinant coefficient (R^2) were 0.9923 which indicated that the polynomial model equation had a high quality fit, a good precision and reliability. Response surfaces were plotted by Design Expert software to explain the interactions of the variables and to determine the optimal level of each variable for the maximum response. Three-dimensional response surfaces were shown in Figs. 2～3. Each figure showed the effects of two factors on the the yield of erinacine while the other one was kept at zero level. The 3-D plot in Figs. 6, which set the extraction time at zero level,showed that the yield of erinacine increased with increasing of liquid/material ratio and extraction temperature at the initial stage and then slightly decreased. Figs. 7 showed the3-D plot at varying liquid/material ratio and extraction time. The3-D plot based on independent variables extraction temperature and extraction time were shown in Figs. 8, while the liquid/material ratio was kept at zero level.

The analysis of response surface was performed by Design Expert software to determine the optimal extraction conditions. The optimal extracting conditions were liquid/material ratio 31.52mL/g, extraction temperature 62.12 ℃ , extraction time 30.47min, respectively. The maximum predicted yield of erinacine was 3.39%.

As the actual operation convenience was considered, the best extraction conditions for erinacine were changed for liquid/material ratio 32 ml/g, extraction temperature 62 ℃, the extraction time for 30 min. Under the condition of the correction, the actual measured extraction yield of erinacine was 3.28%.

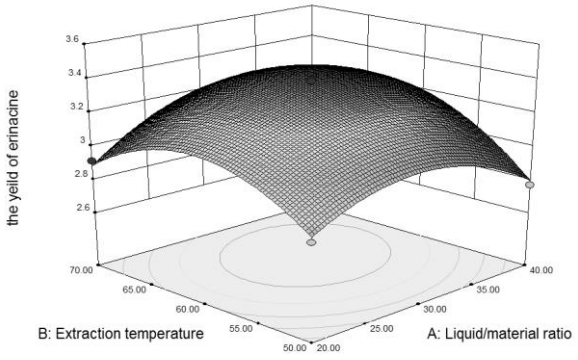

Figure 6. Response surface polts showing the effects of liquid/material ratio and extraction temperature on yield of the erinacine

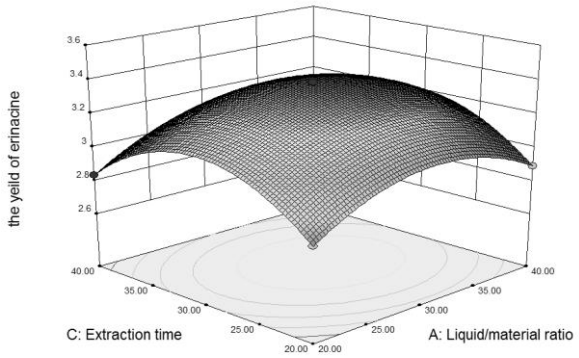

Figure 8. Response surface polts showing the effects of liquid/material ratio and extraction time on yield of the erinacine

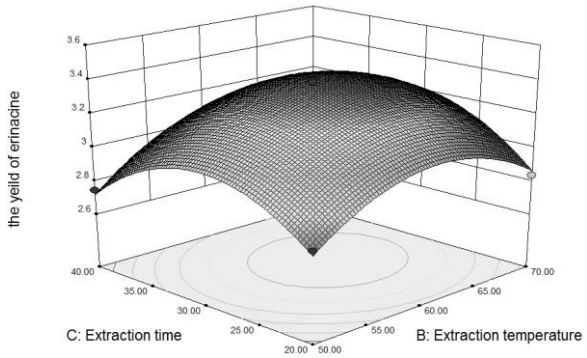

Figure 8. Response surface polts showing the effects of extraction temperature and extraction time on yield of the erinacine

B. Results

The pretreatment of enzymatic hydrolysis whose quality was one percent of the H. erinaceus and acid hydrolysis which was used to adjust pH to 2 was the key technology for improving the extraction yield of erinacine by the method of industrial alcohol reflux extraction. Significantly the extraction yield of erinacine improved from 2.4% which was extracted just by the method of industrial alcohol reflux extraction to 3.2% which was extracted by the method of industrial alcohol reflux extraction with pretreatment of enzymatic and acid hydrolysis. The influence of parameters in sequence for the yield of erinacine were extraction temperature，liquid/material ratio，extraction time. The actual extraction conditions for erinacine was liquid/material ratio 32mL/g,extraction temperature 62℃, extraction time 30min. Under the condition of the correction, the actual measured extraction yield of erinacine was 3.28%. The extraction process obtained by response surface methodology was stable and reasonable, accurate and reliable.The improve of the yield of erinacine will make full use of the resources and provide a development prospect of the industrial preparation of erinacine.

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