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Process optimization in the extract of perilla seed oil with plant protein hydrolysate complex enzyme

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Abstract

Perilla seed oil riched in the ω-3 fatty acid especially α-linolenic acid, is associated with a beneficial role in human health. In the present study, Aqueous Enzymatic Extraction (AEE) of crude oil from Perilla seeds was investigated. The extraction parameters of Perilla seed oil including enzyme type, solid-to-water ratio, pH, temperature, enzyme loading and hydrolysis time were determined by Response Surface Methodology (RSM). The RSM results showed the optimum extraction enzyme for Perilla oil was plant protein hydrolysate complex enzyme. The optimized values of solid-to-water ratio, pH, temperature, enzyme loading, and hydrolysis time for extracting oil from perilla seeds respectively were 1:4, 6, 55 °C, 2% and 4.5 h. Under the conditions, the maximum oil recovery of perilla oil was 88.52%, perilla seed oil extracted by AEE possessed a high extraction yield.

Keywords: perilla seed oil; aqueous enzymatic extraction; Response Surface Methodology (RSM).

Practical Application: Perilla seed oil is rich in omega-3 fatty acids, especially alpha-linolenic acid, which has anti-inflammatory, antioxidant, lipid-lowering, anti-cancer and other diseases. The aim of this study was to establish an efficient and environmentally friendly extraction method of Perilla seed oil. The recovery of oil was greatly improved by aqueous enzymatic extraction. The highest recovery rate of Perilla oil was 88.52%.

1 Introduction

Perilla (*Perilla frutescens L.*), a kind of edible herb which grows annually, belongs to the Lamiaceae family (Asif, 2011). Perilla is one of the most important oilseed crops, widely consumed as a source of edible vegetable oil, and treated as medicine and food homology in Asian countries such as Korea, China, and Japan (Igarashi & Miyazaki, 2013). Its seed oil has a naturally high content of ω-3 fatty acid (53.6%-64%), which confers that Perilla seed oil has a ability to protect people from multiple diseases, such as anti-inflammation, antioxidant, reduce blood fat, and anticancer effects (Hong et al., 2011; Lee & Song, 2012; Povilaityee & Venskutonis, 2000; Wei et al., 2013; Okuno et al., 1998; Zhao et al., 2021; Dhyani et al., 2022). Moreover, Perilla oil can ameliorate Nonalcoholic Fatty Liver Disease in Rats by enhancing fecal cholesterol and bile acid excretion (Chen et al., 2016). On account of perilla oil medicinal properties and nutrient properties, it's relevant to develop methods for the extraction of perilla oil.

The existing techniques used for oil extraction from seed of different origins include soxhlet extraction, organic solvent extraction, cold-pressing, aqueous enzymatic extraction and supercritical carbon dioxide (Jia et al., 2013; Ahmed et al., 2019; Jung et al., 2012; Yusoff et al., 2015; Hao et al., 2021; Wei et al., 2021). Soxhlet extraction takes a long time and consumes a lot of solvents (Mohammadpour et al., 2019; Castro & Priego-Capote, 2010). Extraction with hexane and other organic solvents is cost-effective and the recovery rate is above 95% (Li et al., 2006; Sekhon et al., 2015; Li et al., 2015). But there are some problems such as unsafe operation and environmental pollution. The oil yield of pressing method is low, and the residual oil in the cake is large. Therefore, Aqueous Enzymatic Extraction (AEE), with the characteristics of environmental friendliness and high oil nutrition, is a promising method. Aqueous enzymatic extraction is a new technology to extract oil and protein from most oilseed kernels (Jiang et al., 2010). The enzymes have high efficiency and specificity, hydrolyze and break the cell wall components of oilseed kernels, and promote the release of intracellular oil (Ranalli & Mattia, 1997). Several enzymes used in aqueous enzymatic extraction have been reported, such as protease, α-amylase, cellulase, pectinase and so on. The oil recovery of 81.74% was achieved in cellulase hydrolysis ultrasonic pretreated perilla seed kernel powder (Li et al., 2014). The rapeseed slurry was treated with a combination of pectinase, celulase and β-glucanase at a concentration of 2.5% (v/w), and the recovery rate of free oil was 73-76% (Zhang et al., 2007). The recovery rate (96.0%) was higher by squeezing soybean flakes when protease was used (Jung et al., 2009). As far as we know, the extraction of Perilla seed oil by plant protein hydrolysate complex enzyme has not been reported.

The purpose of the present study is to develop an efficient and environmental friendly method for the extraction of perilla

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seed oil. The effects of enzymatic parameters and types of enzyme on oil recovery were investigated by single factor experiment and RSM design. The recovery of oil was greatly improved by hydro-enzymatic extraction.

2 Materials and methods

2.1 Materials

Chinese herbal perilla seeds (oil content: 30.0%) purchased from Shang shan Ruoshui Chinese Medicinal Materials Co., Ltd., Meizhou City. Plant protein hydrolysate complex enzyme was provided by Henan Qiao Hand Food Additive Co., Ltd. The α-amylase was provided by Xing tai, China. Acid cellulase was purchased from Hunan Xin hongying Biological Engineering Co., Ltd. All the reagents used in the experiment are pure grade analysis, purchased from Chengdu Kelon Co., Ltd.

2.2 Perilla seed kernels preparation

Remove impurities from perilla seeds. Perilla seeds through a 10-20 mesh sieve, in the electric blast drying oven for about 24 h, reserve. Then the seeds were crushed by a small laboratory pulverizer (YGJ Series Roller Mill), which purchased from Jiangyin Hongda Powder Equipment Co., LTD. The crushed perilla seed kernels powder was stored at a reagent bottle.

2.3 Determination of total oil content by Soxhlet extraction

The Soxhlet extraction method can determine the oil content of vegetable oil accurately (Xu et al., 2013). About 10 g of crushed perilla seed kernels powder was weighed by electronic scales, placed in soxhlet extractor. The oil seed extraction was performed for 6 h with 0.3 L of petroleum ether on a water bath at 80 °C. After extraction, petroleum ether was removed under reduced pressure using a vacuum rotary evaporator at 40 °C. The oil recovery in the perilla seeds can be calculated by the using the following equation (Equation 1).

$$Oil\ recovery\ (\%) = Weight\ of\ extracted\ oil\ (g)\ /\ Total\ weight\ of\ oil\ in\ perilla\ seed\ kernel\ sample\ (g)\ x100\% \quad (1)$$

2.4 Aqueous enzymatic extraction of perilla seed oil

Perilla seed powder was extracted by hydroenzymatic method. About 5 g of perilla seed powder were weighted and placed in a 50 mL conical tube. It was then mixed with water in a ratio of 1:4 (sample/water) (Xu et al., 2012). The pH of the mixture was adjusted to the optimum value of each enzyme with 0.5N NaOH and 0.5N HCl. Enzymes (plant protein hydrolysate complex enzyme, α-amylase and acid cellulase) were then added in the amounts recommended by the manufacturers. After enzymatic hydrolysis, the reactants were inactivated for 10 min and stopped in a water bath at 80 °C. Then add n-hexane and stir for 3-4 times to extract perilla oil. The suspension was centrifuged at 4000 g for 15 min at room temperature to obtain oil layers. Finally, n-hexane was recovered by a rotary evaporator. The control group was treated with the same method except the addition of enzyme. The recovery factor was calculated according to the Equation 1.

2.5 Single factor experiment

Prior to RSM, the optimum reaction of three enzymes was evaluated through single factor experiment. According to different enzymes (plant protein hydrolysate complex enzyme, α-amylase and acid cellulose), hydrolysis time (1.5, 3.0, 4.5, 6.0, 7.5 h), enzyme loading (0.5%, 1.0%, 1.5%, 2.0%, 2.5%), pH (2, 4, 6, 8, 10) and enzymolysis temperature (35, 45, 55, 65, 75 °C) were investigated as the key factors.

2.6 Extraction of perilla oil by enzyme and compound enzyme respectively

In order to compare the effects of three enzymes (acid cellulase, plant protein hydrolysate complex enzyme and α-amylase), perilla oil was extracted under the same conditions of enzyme dosage, pH, temperature, time and solid-liquid ratio. Under the conditions of enzyme dosage 2%, pH 5, temperature 55 °C and time 4.5 h, the extraction rate of oil was determined. The extraction effects of single enzyme and compound enzymes was also investigated.

2.7 Response Surface Methodology (RSM) design and statistical analysis

Based on the results of single experiments, plant protein hydrolysate complex enzyme was selected for subsequent experiments. Response Surface methodology (RSM) was designed to determine the optimal extraction condition on three factors (a: enzyme dosage, b: pH, c: enzymolysis temperature) and three levels. The solid-to-water ratio (4:1) and hydrolysis time (4.5 h) were fixed as constant variables. The RSM design consisted of 17 independent experiments. Extraction recovery of oil (Y) was used as an index for optimizing conditions. The run order, variable conditions, and experimental and predicted values are shown in Table 1A.

Data were analyzed by ANOVA to investigate lack of fit and the effects of linear, quadratic, and interaction variables on perilla oil extraction. Data analysis and RSM were performed with Design Expert software program (Version 10). Design Expert was a rich experimental analysis software.

2.8 Characterization of perilla oil

The lead in perilla oil was determined by the method of vegetable oils inspection for determination of foreign matter (GB/T 5529). The total arsenic was determined by national standard for food safety determination of total arsenic and inorganic arsenic in food (GB 5009.11). The benzo (α) pyrene was determined by determination of benzo (α) pyrene in food (GB/T 5009.27). Acid value (In fat) (KOH), peroxide value (In fat) and leaching residual oil solvent were detected by methods for analysis of hygienic standards for edible vegetable oils (GB/T 5009.37). Animal and vegetable fats and oils were determined by determination of Lovibond colour (GB/T 22460). Impurities and moisture respectively was checked by animal and vegetable oil inspection (GB/T 5529; GB/T 5528). The aflatoxin (AFTB1) was determined by determination of aflatoxin in food (GB/T 18979). Linolenic acid was checkouted by animal and vegetable

Table 1. Box-Behnken design and the response surface methodology test results.

A. Box-Behnken design and extraction yield of oil					
Run	Factor 1a: enzyme dosage (%)	Factor 2b: pH	Factor 3c: enzymolysis temperature (°C)	Extraction yield of oil (%)	
				Actual value	Predicted value
1	2.5	6	45	54.08	51.79
2	2	6	55	85.56	86.09
3	2.5	6	65	62.27	64.38
4	2	8	65	60.70	58.53
5	2.5	8	55	47.69	47.76
6	2	4	65	56.24	54.00
7	2.5	4	55	46.70	46.81
8	2	8	45	36.46	38.69
9	1.5	8	55	34.35	34.24
10	1.5	4	55	39.77	39.71
11	2	6	55	88.53	86.09
12	2	6	55	88.52	86.09
13	2	6	55	83.32	86.09
14	1.5	6	45	43.13	41.01
15	2	6	55	84.52	86.09
16	2	4	45	45.57	47.74
17	1.5	6	65	52.23	54.53

B. The response surface methodology test results					
Source	Sum of squares	df	Mean square	F value	p-value probe > F
Model	5916.13	9	657.35	75.32	< 0.0001
a	212.71	1	212.71	24.37	0.0017
b	10.27	1	10.27	1.18	0.3139
c	340.68	1	340.68	39.04	0.0004
ab	10.29	1	10.29	1.18	0.3136
ac	0.21	1	0.21	0.024	0.8805
bc	46.08	1	46.08	5.28	0.0552
a^2	1750.08	1	1750.08	200.54	< 0.0001
b^2	2339.93	1	2339.93	268.13	< 0.0001
c^2	687.29	1	687.29	78.75	< 0.0001
Residual	61.09	7	8.73		
Lack of fit	38.88	3	12.96	2.33	0.2153
Pure error	22.21	4	5.55		
Cor total	5977.21	16			
R^2	0.9898				
Adj.R^2	0.9766				

P < 0.0001 highly highly significant; p < 0.01 highly significant; 0.05 < p < 0.01 significant; p > 0.05 not significant.

oils-methyl fatty acids-gas chromatography (GB/T 17377). All indexes were determined according to Chinese national standard method for analysis of hygienic standard of edible oils.

3 Results and discussion

3.1 Oil recovery of perilla by Soxhlet extraction

Soxhlet extraction, which is the most referenced technique for evaluating the performance of solid-liquid extraction methods (Danlami et al., 2014), can be used to determine of oil content of vegetable oil. The average oil content of perilla seed measured by Soxhlet extraction method was 29.70%. In this experiment, the content of perilla oil extracted by Soxhlet extraction is considered as the total oil content.

3.2 Extraction rate of perilla seed by a single factor test of aqueous enzymatic method

Aqueous enzymatic extraction method utilizes enzymes to aggravate the destruction of the cell biological membrane and enhance the oil extracted rate (Jiang et al., 2010). In this study, three enzymes including acid cellulase, plant protein hydrolysate complex enzyme and α-amylase were selected as enzyme preparations for extract perilla seed oil. The single factor experiments of enzymatic hydrolysis time, enzyme dosage, and pH value and enzymolysis temperature were carried out. The results of perilla oil extracted by three enzymes separately in aqueous enzymatic method were shown in Figure 1.

As shown in Figure 1A(a), when acid cellulase hydrolysis time was 4.5 h, the extraction rate of perilla oil was the highest.

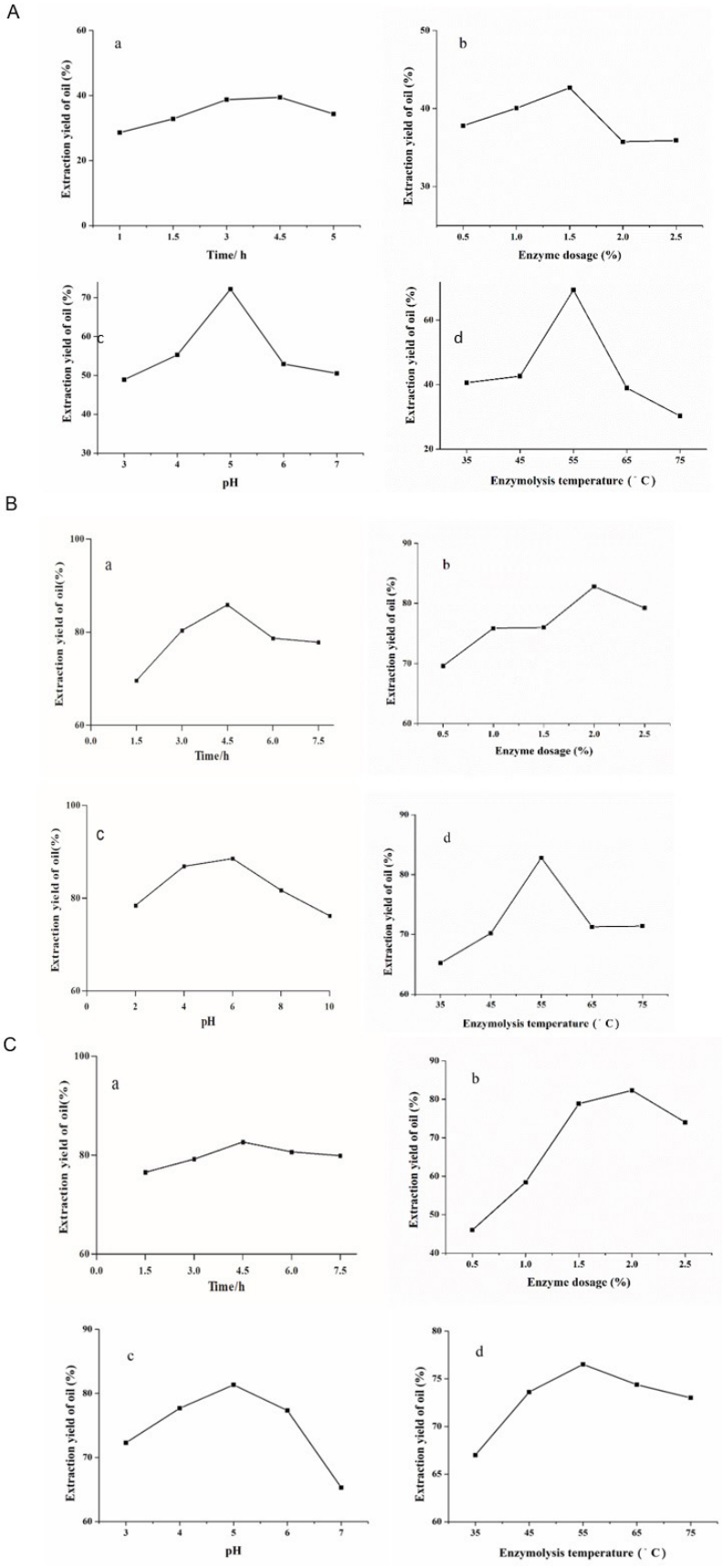

Figure 1. Oil yield of a single factor experiment with three enzymes. (A) acid cellulose hydrolysis, (B) plant protein hydrolysate complex enzyme, (C) α-amylase hydrolysis.

Similarly, controlling the other factors, when the enzyme dosage was 1.5% or pH was 5, the extraction rates of perilla oil were also the highest [Figure 1A(b), Figure 1A(c)]. Extraction recovery increased with increasing extraction temperature up to 55 °C, followed by a significant enhancement in oil recovery [Figure 1A(d)]. Therefore, the best extraction temperature was 55 °C. Taken together, the optimal conditions for the extraction of perilla seed oil by acid cellulase were as follows: enzyme hydrolysis time, 4.5 h; enzyme dosage, 1.5%; pH = 5; enzymolysis temperature, 55 °C, and oil extraction rate was 72.20%. The data showed in Figure 1B(a), from 1.5 to 4.5 h of extraction time of plant protein hydrolysate complex enzyme, oil recovery increased, reaching a maximum at 4.5 h. And when the enzyme dosage was 2%, pH was 6, and the enzymolysis temperature was 55 °C, the extraction rate of perilla oil was superior [Figure 1B(b)-1B(d)]. In a result, the optimal conditions for the extraction of perilla seed oil by plant protein hydrolysate complex enzyme were as follows: the enzyme hydrolysis time was 4.5 h, the enzyme dosage was 2%, pH was 6.5, the enzymolysis temperature was 55 °C, and oil extraction rate was 88.52%. When enzymatic hydrolysis time of α-amylase was 4.5 h, the extraction rate of perilla oil was higher [Figure 1C(a)]. The results in Figure 1C(b) exhibited that the oil recovery increased first, and then, decreased with the improve of enzyme dosage from 0.5% to 2.5%, reaching a maximum at 2.0%. The effects of pH and enzymolysis temperature on oil recovery were shown in Figure 1C(c)-1C(d). Resultly, the optimum conditions for the extraction of perilla seed oil by α-amylase were as follows: the enzyme hydrolysis time was 4.5 h, the enzyme dosage was 2%, pH was 5, the enzymolysis temperature was 55 °C, and oil extraction rate was 82.66%.

3.3 Extraction rate of perilla seed by a individual enzyme and combined enzyme

It was necessary to add enzymes in aqueous enzymatic method to improve the extraction efficiency of perilla seed oil. As shown in Table 2, the highest oil recovery of perilla seed was obtained when plant protein hydrolyzed complex enzyme was used (84.30%), followed by α-amylase (82.66%) and acid cellulose (35.75%). The present study investigated that plant protein hydrolysate complex enzyme gave a higher oil recovery, which conformed to the early observation that cellulose and amylase were less effective than neutrase in oil extraction from wheat germ and *Jatropha curcas L.* seed kernels (Li et al., 2010; Shah et al., 2005). Due to the presence of protein as a major component in the cell wall of perilla seeds, the oil is released more easily from the cellular matrix by degrading the proteins, which is achieved by the action of protease, and it was consistent with the study of perilla seed oil and peanut oil extraction (Yuan et al., 2019; Jiang et al., 2010). Plant protein hydrolyzed complex enzyme, as a kind of proteases, was more suitable to be used in aqueous enzymatic extraction of perilla seeds. At the same time, the enzymes were combined to evaluate their cooperative effects on extracting the oil. Under the conditions of the enzyme hydrolysis time 4.5 h, the enzyme dosage 2%, pH 5, and the enzymolysis temperature 55 °C, the application of a mixture of plant protein hydrolyzed complex enzyme, α-amylase and acid cellulose to extract perilla seed oil increased the perilla seed oil recovery. However, the recovery rate of perilla seed oil was not as high as that of plant protein hydrolyzed complex enzyme alone. Coincident with our result, a similar outcome was reported that a mixture of cellulase and protease did not significantly improve the soybean oil recovery from individual cellulose (Lamsal et al., 2006).

3.4 Second-order polynomial model

Perilla oil extraction rate based on the RSM design and the ANOVA results were shown in Table 1. The model F-value of 75.32 and low P ($p < 0.0001$) implied that the model was statistically significant (Table 1B). Perilla oil extraction rate was remarkably affected by enzyme dosage and temperature, but the effect of pH was non-significant. There is only a 0.01% chance that an F-value this large could occur due to noise. The enzyme dosage impacts the extent of an interaction between the enzyme and substrate molecules, and temperature can affect all chemical reactions including enzyme-catalyzed reactions. A change in pH may alter the enzyme activity but a few tolerate large changes in pH (Jiang et al., 2010). The coefficient of determination (R2) of the model was 0.9898, manifesting that 98.98% of the experimental oil extraction rate values matched the model-predicted values. Study reported that model was appropriate when $R^2 > 0.75$ (Man et al., 2010). And the "lack of fit F-value" of 2.33 indicated this value was not significant. Lack of fit value ($p > 0.05$) also revealed that the model was suitable. The second-order polynomial (Equation 2) was fitted using software.

Table 2. Extraction yield of perilla oil by different enzyme.

Enzyme	Perilla seed weight/g	Enzyme dosage/%	pH	Enzymatic hydrolysis temperature/°C	Time/h	Perilla oil weight/g	Extraction rate of oil/%
A	5.0024	2	5	55	4.5	0.5365	35.75
B	4.9936	2	5	55	4.5	1.2629	84.30
C	5.0001	2	5	55	4.5	1.2400	82.66
2%A + 2%B	4.9956	2	5	55	4.5	1.0166	67.86
2%A + 2%C	5.0008	2	5	55	4.5	0.8442	56.35
2%B + 2%C	5.0019	2	5	55	4.5	1.0136	67.66
2%A + 2%B + 2%C	4.9987	2	5	55	4.5	1.0981	73.30

A: Acid cellulase; B: Plant protein hydrolysate complex enzyme; C: α-amylase.

$$Y = 86.09 + 5.16a - 1.13b + 6.53c + 1.60ab - 0.23ac + 3.39bc - 20.39a^2 - 23.57b^2 - 12.78c^2 \quad (2)$$

Where, Y is the response variable (extraction rate of oil), while a, b and c, stand for enzyme dosage, pH and temperature, respectively.

3.5 RSM analysis

There were three-dimensional (3D) response surface graphs and two-dimensional (2D) contour plots (Figure 2). Response surface graphs are effective to determine the maximum, minimum, and middle points of the response. The three-dimensional (3D) response surface graphs showed that the influence of pH, enzyme dosage and temperature was more significant with increasing slope. The condition corresponding to the center of two-dimensional (2D) contour plots was the optimal extraction method. Figure 2A showed the effects of enzyme dosage and pH levels on oil extraction rate. The closer the enzyme dosage was to 2% and the closer the pH was to 6, the oil extraction rate was higher. As a result, moderating enzyme dosage and pH levels resulted in a high extraction recovery. The effects of

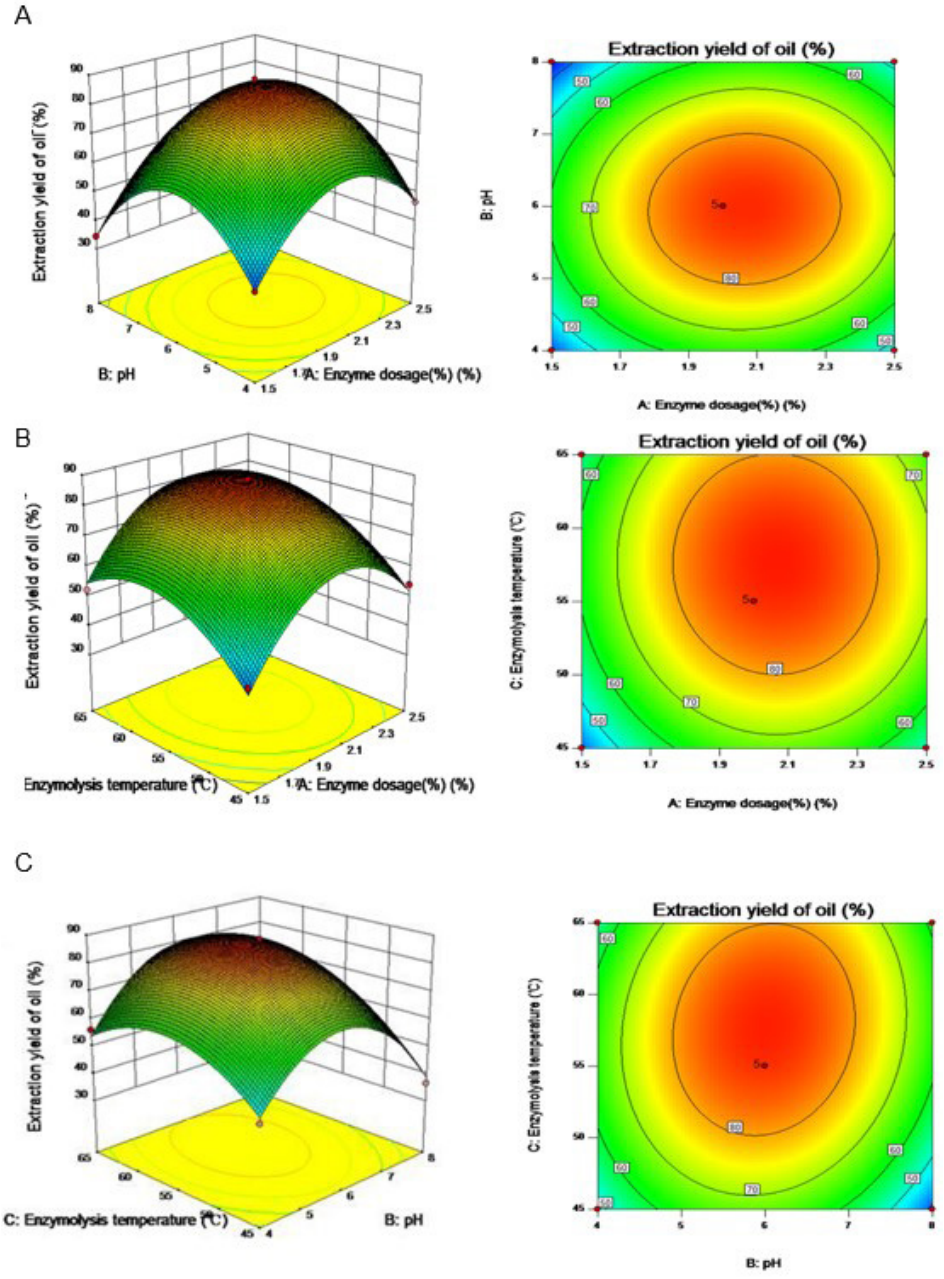

Figure 2. Response surface and contour plot for the extraction rate of oil under different conditions. (A) a function of enzyme dosage and pH at a regular enzymolysis temperature of 55 °C, (B) a function of enzyme dosage and temperature at a regular pH of 6, (C) a function of temperature and pH at a regular enzyme dosage of 2%.

Table 3. Characterization of perilla oil and the national standard.

Physicochemical properties	The actual value by plant proteolytic mixed enzyme extracted	Scope of state regulation
Lead (mg/Kg)	not detected	≤ 0.1
Total arsenic (mg/Kg)	not detected	≤ 0.1
Benzoa-(α)-pyrene (g/Kg)	not detected	≤ 10
Acid value (In fat) (KOH) (mg/g)	1.5	≤ 3
Peroxide value (In fat) (g/100 g)	0.055	≤ 0.25
Leaching residual oil solvent (mg/Kg)	not detected	≤ 50
Colour and lustre (Lovibond color slot)	yellow 20 red 8	Yellow ≤ 40 Red ≤ 15
Impurities (g/100 g)	0.03	≤ 0.1
Moisture (g/100 g)	0.012	≤ 0.3
AFTB1(g/Kg)	< 10	≤ 10
Linolenic acid (g/100 g)	63.2	≥ 50

The detection limit of lead is 5 g/Kg; the detection limit of total arsenic is 0.01 mg/Kg ; the detection limit of benzo (α) pyrene is 0.5g/Kg; the detection limit of solvent residue is 0.10 mg/Kg.

enzyme dosage and temperature levels on oil extraction rate were indicated in Figure 2B. The oil recovery increased with increasing temperature up to 55 °C. This illustrated the influence of pH and temperature on oil extraction rate (Figure 2C). Highest oil recovery was observed in the case of 55 °C and pH 6. In conclusion, the best condition of oil recovery was obtained with enzyme dosage of 2%, hydrolysis temperatures of 55 °C, and pH of 6, and the maximum oil recovery of perilla oil was 88.52%.

3.6 Characterization of perilla oil analysis

The lead, total arsenic, benzoa-(α)-pyrene and leaching residual oil solvent have not been detected (Table 3). Acid value (In fat) (KOH) was 1.5 in perilla oil, not exceed the specified test line. Peroxide value (In fat) (g/100 g) was 0.055. Color and lustre (Lovibond Color slot) of yellow was 20 and red was 8. Impurities (g/100 g), moisture (g/100 g) and linolenic acid (g/100 g) respectively was 0.03, 0.012, 63.2 in perilla oil. Finally, aflatoxin was less than 10 μg/kg. All the indexes were in line with the national food safety standards of the People's Republic of China, which proved that the quality of the aqueous enzymatic extraction of perilla seed oil is qualified.

4 Conclusion

In this study, the optimum extraction conditions of perilla oil in aqueous enzymatic method was obtained by the way of RSM, which was in the case of individual plant protein hydrolysate complex enzyme, an enzymolysis temperature of 55 °C, pH at 6, enzyme dosage of 2%, an enzymolysis time of 4.5 h and a liquid-to-solid ratio of 4:1. Under these conditions, the maximum extraction rate of perilla seed was 88.52%. Interestingly, the application of plant protein hydrolysate complex enzyme was significantly higher than the combination of the three enzymes in oil recovery. In addition, it had not been reported that plant protein hydrolysate complex enzyme was used in perilla oil by aqueous enzymatic method. In comparison with other oil extraction methods, aqueous enzymatic method markedly improved perilla oil recovery. What's more, the method had the advantages of high efficiency and environmental friendliness.

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