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Producing Defatted Hybrid Catfish with High Protease Activity Via Optimization of Sc-CO_2 Extraction Process

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ABSTRACT

Fish viscera is a great source for recovering polyunsaturated fatty acids (PUFAs), proteins and digestive enzymes. However, the presence of lipids in fish viscera reduces the efficiency of extracting, isolating and purifying the enzymes. Supercritical carbon dioxide (Sc-CO_2), a green solvent, is useful in substituting organic solvent defatting process. Sc-CO_2 helps to remove the lipid in the viscera with minimal denaturing of the proteins and enzymes found in the defatted sample. The viscera of hybrid catfish (*C. gariepinus* × *C. macrocephalus*) in this study were defatted by the Sc-CO_2 extraction process. The protease was then, extracted from the defatted viscera. This study aimed to optimize the pressure and temperature of Sc-CO_2 in the defatted viscera. The optimization of Sc-CO_2 parameters was based on the Central Composite Design (CCD) of Response Surface Methodology (RSM). Optimum points were observed within the variables of pressure (P): 17-25 MPa and temperature (T): 39-43 °C with constant CO_2 flow rate (F) and extraction time (t_{ext}) at 25 g/min and 30 min, respectively. The response variables were executed on the amount of fat residue and protease specific activity in the defatted viscera. The optimum Sc-CO_2 parameters were found to be at P: 18.17 MPa and T: 41.33 °C with the highest protease activity of 77.51 U/mg and fat residue of 71.17 %. The coefficient of determination (R^2) for protease specific activity and fat residue was 90.27% and 93.03%, respectively. The differences between the verification and predicted values for both responses were less than 5%. Hence, the feasible optimum condition to produce a defatted sample with high specific activity at relatively low fat residue as predicted by the RSM in MINITAB statistical software version 16 was acceptable. Nevertheless, the ability of Sc-CO_2 to defat the sample with minimal denaturing proteins and enzymes highlights the usefulness of this technology in substituting other organic solvents for the defatting process.

Keywords: Defatted, hybrid catfish viscera, protease, response surface methodology, supercritical carbon dioxide

INTRODUCTION

The hybrid catfish (*Clarias gariepinus* × *Clarias macrocephalus*) or also known as ikan keli in Malaysia from the genus *Clariidae* is readily accepted by consumers as it resembles the local catfish and fetches a better price (Adan, 2000).

The hybrid catfish inherited major commercially desirable attributes from its parents, for instance, fast growth, high survival rate, resistance to various diseases and the ability to adapt to various environmental conditions (Bunnoy *et al.,* 2022; Ninwichian *et al.,* 2022). The growing population has urged the fish industry to produce more fish products to fulfil consumers' demands and diets (Jamalluddin *et al.,* 2022). To illustrate, the waste generated from the total fish production of 167.2 million metric tonnes (MMT) corresponds to 75.24 MMT globally, equivalent to 45% of the live weight (Murthy *et al.,* 2018). Fish by-products (FBPs) are usually composed of heads (9–12% of total fish weight), viscera (12–18%), skin (1–3%), bones (9–15%), and scales (about 5%) (FAO, 2022). It is often discarded without any attempt of recovery and regarded as waste. Consequently, inappropriate disposal of FBPs is prone to creating an unhygienic environmental atmosphere (Mohanty *et al.,* 2018), thus, generating tonnes of fish by-products (FBPs). FBPs such as heads, viscera, skin, bones and scales are often discarded without any attempt of recovery and regarded as waste. Inappropriate disposal of FBPs is prone to creating an unhygienic environmental atmosphere (Mohanty *et al.,* 2018). Nevertheless, FBPs are great sources for the recovery of enzymes. For instance, fish viscera are rich in digestive enzymes, especially protease (pepsin, trypsin, chymotrypsin, elastase, and collagenase) (Borges *et al.,* 2023; Shahidi and Kamil, 2001). Proteases execute a large variety of functions and have important biotechnological applications either in food industries or non-food industries (detergent, leather and pharmaceutical).

Fish viscera with high fat content tend to make homogenization of the sample difficult and also, complicate the subsequent purification steps of enzymes due to the resulting extract containing a high quantity of finely dispersed lipids (Geethanjali and Subash, 2013). Removal of the lipid layer would attain higher efficiency in extracting, isolating and purifying the enzymes. Conventional methods like solvent extraction (Soxhlet method) have been greatly used and discussed for more than a decade to remove lipid layers in the sample. The method basically involved the use of organic solvents like petroleum ether, hexane, methanol, acetone, chloroform, or dichloromethane at high temperatures (> 78 °C) which is potentially hazardous and toxic and requires an additional step to remove the solvent by using a rotary evaporator (Adeoti and Hawboldt, 2014) makes it unfavourable for human consumption (Sarker *et al.,* 2012), tedious and time-consuming (Al-Jabari, 2002; Melgosa *et al.,* 2020). More importantly, this step can negatively affect the nutritional quality of the fish oils, denature proteins, and degrades the heat-sensitive, labile, and natural compounds in the samples.

Previously, attention has been given to the supercritical carbon dioxide (Sc-CO_2) in extracting essential oil for PUFAs and other bioactive materials only (Ferdosh et al., 2015; Hajeb et al., 2015; Haq et al., 2017; Haq & Chun, 2018; Kuvendziev et al., 2018; Lisichkov et al., 2014; Rubio-Rodríguez et al., 2012; Sarker et al., 2012) but not for defatting purposes and investigating the fat residue left in the sample. According to Jamalluddin *et al.* (2022), there are scarcity of studies on lipid removal using Sc-CO_2 for enzyme recovery although it started as early as 2006. This could be due to insufficient knowledge of handling the delicate fish protein before or during Sc-CO_2 extraction(Jamalluddin *et al.,* 2022). The most recent work on lipid extraction using Sc-CO_2 for enzyme recovery was reported by Asaduzzaman and Chun (2015) and Lamas (2015). It has been reported that the fish trypsin was successfully recovered without significant denaturation after lipid removal at mild Sc-CO_2 conditions such as 25 Mpa and 45 °C for 2.5 h (Chun et al., 2011). Based on Asaduzzaman and Chun (2015), amylase (45 U/mg), trypsin (10 U/mg), and lipase (8 U/mg), exhibited higher activity in defatted mackerel muscle via Sc-CO_2 extraction process compared to hexane extraction process (amylase: 38 U/mg; trypsin: 7.8 U/mg; lipase: 6 U/mg), except for protease (Sc-CO_2 treated sample: 19 U/mg; hexane treated sample: 22 U/mg). The loss of digestive enzyme activity from defatted mackerel muscle via hexane extraction was due to the denaturation of protein in the sample as a result of the long extraction time and the use of organic solvent. Also, this directly implied that the removal of lipids with conventional organic solvents causes protein denaturation (Asaduzzaman and Chun, 2015; Chun et al., 2011; Park et al., 2008). In enzyme recovery studies, 10 to 30% of oil yield managed to be collected after the Sc-CO_2 extraction process to defat the samples such as fish muscle, fish viscera and squid viscera (Asaduzzaman and Chun, 2015; Jamalluddin et al., 2022; Park et al., 2008; Uddin et al., 2009).

As an environmentally friendly alternative to the conventional method, Sc-CO_2 is applied to defat the sample and subsequently, the defatted sample is used for extracting and purifying the enzyme. Sc-CO_2 is non-toxic, non-flammable, inert, and readily available in high purity at low cost. Sc-CO_2 is generally recognized as safe

(GRAS) by the Food and Drug Administration and European Food Safety Authority (Da Silva *et al.,* 2016; Ferrentino *et al.,* 2019) and thus, useful as a substitute for the organic solvent in the defatting process (Chun *et al.,* 2011). Carbon dioxide (CO_2) as an extractant in the supercritical extraction process is easily removed from the extract, thus, producing solvent-free extract, but most importantly approved for food processing without declaration (Brunner, 2005). This study aimed to investigate the optimum pressure and temperature used in the Sc-CO_2 extraction process to defat the hybrid catfish viscera based on the highest amount of protease specific activities at a low amount of fat residue.

MATERIALS AND METHODS

Materials

Fresh hybrid catfish (*C. gariepinus* × *C. macrocephalus*) viscera were collected as soon as the fish breeder eviscerated the fishes at Aquaculture Resource Centre (ARC), Sungai Buloh, Selangor Darul Ehsan, Malaysia. The fresh viscera were packed in a polyethylene bag and placed in the Coleman® handheld box containing ice packs at 4 °C and transported to the research laboratory at the Faculty of Applied Sciences, UiTM Shah Alam, immediately about 25 km distance within 33 min. At the laboratory, the viscera were sorted, weighed, and stored in an airtight glass bottle at -20 °C until further use and analysis. Chemicals used in this experiment were of analytical grade and obtained from Evergreen Engineering and Resources and FC-Bios Sdn. Bhd., Malaysia.

Sample preparation for the Sc-CO_2 extraction process

The viscera of hybrid catfish were dried in a freeze-dryer (Model: SANYO- Biomedical freeze drier) at a constant drying temperature of −47 °C and vacuum at 0.133 bar for about 72 h. The dried samples were crushed using a handheld blender (Russell Taylors Multifunctional Hand Blender HB-6) and sieved (4 mm) by mesh. These samples were then stored at -80°C and used for oil extraction by Sc-CO_2 process (defatting viscera).

Supercritical Carbon Dioxide (Sc-CO_2) extraction

The oil extraction was done in a Supercritical Fluid Extraction Lab Scale Plant apparatus (SCFE) (Deven Supercriticals Pvt. Ltd., India) at the Supercritical Fluid Centre, Universiti Putra Malaysia (UPM). The schematic diagram of the Sc-CO_2 extraction process is shown in Fig. 1. Prior to the Sc-CO_2 process, the chiller (No.4) is cooled at 4 °C while heating the stainless-steel extraction vessel (No.6) to the desired extraction temperature ($T>T_c$). For each trial, 100 g of the freeze-dried sample was placed inside the 45 μm nylon sachet. The sachet containing the sample was inserted and placed at the bottom of the 1 L stainless steel extraction vessel. The extraction vessel was plugged with a cap. CO_2 was pumped into the vessel by a high-pressure pump (No. 3) until achieving the desired pressure ($P>P_c$). A back pressure regulator (BPR) was used to control the pressure of CO_2. The vessel temperature was maintained by the heater. Once the process reached $P>P_C$ and $T>T_C$, the supercritical carbon dioxide (Sc-CO_2) was produced and acted as an extraction solvent. The Sc-CO_2 and fish oil extract (Sc-CO_2 + fish oil extract) were rapidly depressurized by the metering valve (No.15) to reduce the pressure of CO_2 before being emitted to the surroundings. After Sc-CO_2 extraction, the fish oil extract can be collected from the separating vessel (No. 7) whereas the defatted fish viscera in the extraction vessel (No. 6) were stored at −20 °C prior to further analysis. The hybrid catfish viscera were extracted at temperature and pressure ranges of 39-43 °C and 17-25 MPa, respectively (Table 1). The effect of pressure and temperature on the protease specific activity of the hybrid catfish viscera and the fat residue in the defatted sample was evaluated based on response surface methodology (RSM). The flow rate of CO_2 and time of extraction was kept constant throughout the extraction process at 25 g/min and 30 min, respectively. The optimum temperature and pressure were selected based on the highest protease specific activity recorded. The defatted sample at optimum conditions was stored at -20 °C until further use and analysis.

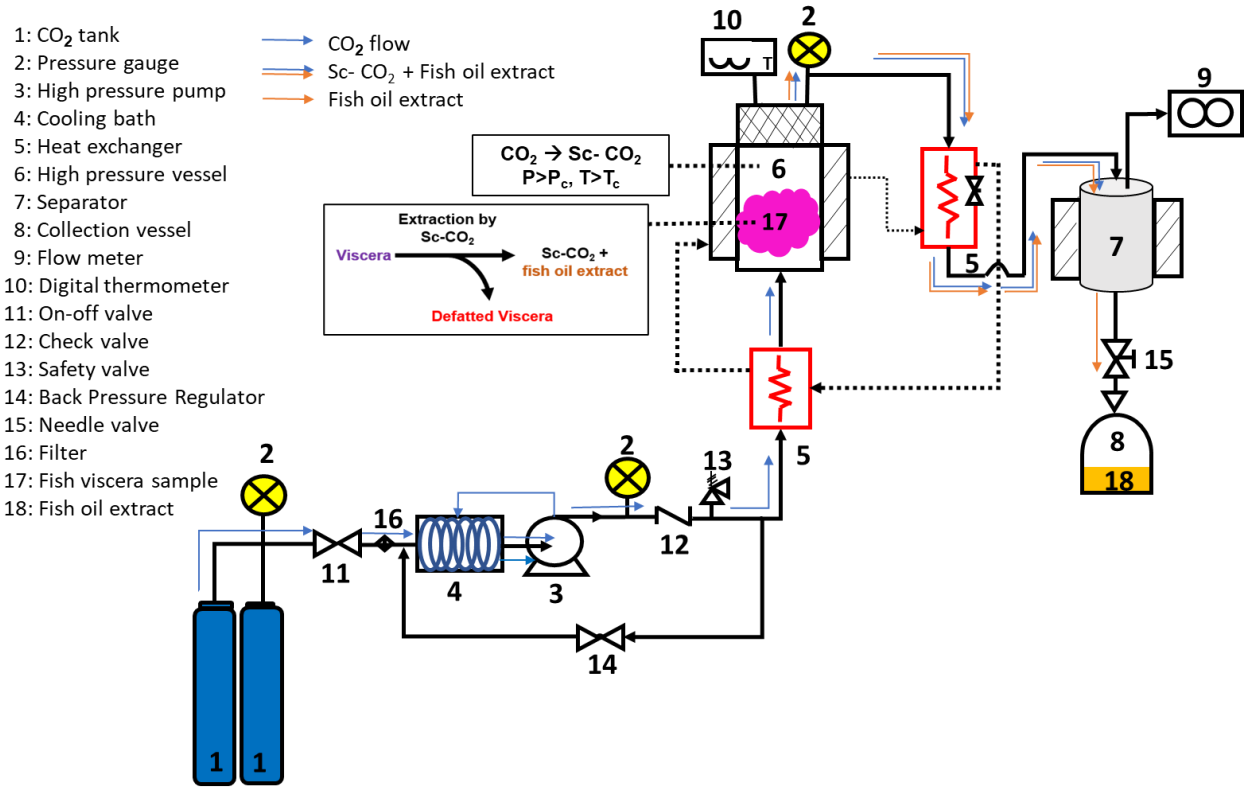

Fig. 1. The schematic diagram of Sc-CO_2 for defatting the catfish viscera (No. 17).

Experimental design

A central composite design (CCD) consisting of 13 experimental runs with five replications at the central points was employed in this study using Response Surface Methodology (RSM). The experimental design was used to optimize two Sc-CO_2 independent variables namely pressure (X_1) and temperature (X_2). Optimally high proteolytic activity (Y_1) and low fat residue (Y_2) of the defatted sample were measured as responses to the independent variables.

Table 1. The coded and uncoded values used in RSM for the optimization of the Sc-CO_2 parameters.

Factors	Codes	Levels				
		-α	-1	0	+1	+α
Pressure (MPa)	X_1	17.0	19.0	21.0	23.0	25.0
Temperature (°C)	X_2	39.0	40.0	41.0	42.0	43.0

A full second-order polynomial model of the design was applied to evaluate the response variables (Y_1 and Y_2) of defatted viscera as a function of the independent variables (X_1 and X_2) as well as their interactions. The second-order polynomial model equation used in the response surface analysis is presented in the equation below:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_1^2 + \beta_4 X_2^2 + \beta_5 X_1 X_2 + \varepsilon \qquad \textbf{Eqn. 1}$$

where Y represents the predicted response variables, X_1 and X_2 are independent variables in coded values, β_0 is the value of the fitted response variables at the central point of the experiment (constant), β_1 and β_2 are linear coefficients, β_3 and β_4 are square (quadratic) coefficients, β_5 is interaction coefficients and ε is the random error.

RSM verification process

The optimum condition predicted by the MINITAB statistical software version 16 was used to repeat the Sc-CO_2 process. The differences between the verified value and predicted value must be less than 5% for proteolytic activity and fat residue.

Soxhlet extraction

The total lipid content of the defatted viscera was determined by the Soxhlet extraction method. In this study, total lipid content (fat content) represents the fat residue found in the defatted viscera after the Sc-CO_2 process. Five grams of finely ground dried samples were extracted with 170 mL of petroleum ether (boiling point: 40/60) over 8 h of extraction period. The excess water and petroleum ether residue in the extracted oils was evaporated using a rotary evaporator (Heidolph, Germany) at a temperature of 45 °C. The evaporated sample was then dried in an oven at 105 °C for 1 hr. The percentage of oil yield (%) was calculated using the following equation:

$$\text{Fat content (\%)} = \frac{(W_2 - W_1)}{S} \times 100\% \qquad \textbf{Eqn. 2}$$

Where W_1 is the weight of the empty round bottom flask (g), W_2 is the weight of the round bottom flask with extracted fat (g) and S is the weight of the sample (g).

Extraction of crude protease

Crude protease from defatted viscera samples was extracted at temperatures not exceeding 4 °C within 24 hrs. The viscera (10 g) were homogenized in a homogenizer (T25 Digital Ultra Turrax Homogenizer, Germany) for 5 mins in 50 mL extraction buffer A (10 mM Tris-HCl pH 8.0, containing 1 mM $CaCl_2$) at 4 °C at the 1:5 (w/v) viscera to buffer ratio. The mixture was centrifuged (Eppendorf 5415 R Refrigerated Centrifuge, Germany) at 10,000 rpm for 10 mins at 4 °C. Then, the supernatant was collected whereas the pellet was discarded. The supernatant was used as the crude protease and stored at -20 °C within a month.

Protein concentration

Protein concentration was determined by the Bradford method (Bradford, 1976) with slight modifications. Bovine serum albumin (BSA) in the range of 0-2 mg/mL was used as the standard and measured at 595 nm (Thermo Fisher Scientific Spectrophotometer Helios Zeta, USA) to estimate the protein concentration of the samples.

Proteolytic activity assay

Proteolytic activity was assayed using the casein digestion unit (CDU) method (Enzyme Development Corporation, 2015) with slight modification. Approximately, 5 mL of 0.65% (w/v) casein solution in 50 mM of disodium phosphate buffer (pH 7) was used as a substrate. Initially, the substrate was heated for 10 mins at 37°C. Then, the crude protease solution (1 mL) was mixed with the substrate and incubated for 10 mins at 37°C. As for the blank (E_b), distilled water was used instead of crude protease solution. Trichloroacetic acid (TCA) solution was prepared using 110 mM TCA powder, 365 mM sodium acetate and 330 mM of acetic acid. At exactly 10 min., the TCA solution (5 mL) was used to stop the reaction and incubated for 30 mins at 37°C. After 30 min., the solution was cooled at room temperature for 10 min. The sample was subjected to filtration using a 0.45 μm polyethersulfone (PES) syringe filter. The supernatant (E_a) was measured at 275 nm (IMPLEN

NanoPhotometerTM UV-VIS spectrophotometer, serial no. 1361, version 7122 V1.6.1, USA). A standard tyrosine solution was prepared containing exactly 50μg/ml of L-Tyrosine in 0.1 N HCl. The absorbance of the standard tyrosine solution was read and recorded at 275 nm (E_s) using distilled water as blank. One unit of protease activity is defined as the amount of enzyme required for liberating 1 μmol of tyrosine per minute from casein. The protease total activity was determined according to Ismail and Jaafar (2018) by the following formula:

$$\text{Protease total activity} = \frac{(E_a - E_b)}{E_s} \times \text{concentration of standard tyrosine} \times \frac{V_r}{T_r} \times \text{DF} \qquad \textbf{Eqn. 3}$$

Where E_a is the absorbance of the sample, E_b is the absorbance of the blank, E_s is the absorbance of standard (tyrosine), V_r is the reaction volume, T_r is the reaction time and DF is a dilution factor.

Protease specific activity

The specific activity of the protease was calculated by the following formula:

$$\text{Specific activity } \left(\frac{U}{mg}\right) = \frac{\text{Enzyme activity } \left(\frac{U}{mL}\right)}{\text{Total protein } \left(\frac{mg}{mL}\right)} \qquad \textbf{Eqn. 4}$$

Statistical analysis

All measurements were carried out in triplicate. Statistical analysis for analysis of variance (ANOVA), the multiple regression analysis and the response surface regression were carried out using MINITAB statistical software version 16 at a 95% confidence level ($p < 0.05$).

RESULTS AND DISCUSSION

Optimization of Sc-CO_2 extraction conditions on the protease specific activity and fat residue

In this study, the viscera of hybrid catfish (*C. gariepinus* × *C. macrocephalus*) were defatted priorly via the Sc-CO_2 process. The protease was then, extracted from the defatted viscera. Optimization of the Sc-CO_2 extraction process was conducted with protease specific activity and fat residue left in the defatted sample as response variables and was executed against two independent variables of pressure (P) and temperature (T) of the Sc-CO_2 extraction process. The Sc-CO_2 extraction process was carried out to extract the lipid from the hybrid catfish viscera at different pressures (17.0-25.0 MPa) and temperatures (39-43 °C) with constant CO_2 flow rate (F) and extraction time (t_{ext}) at 25 g/min and 30 min, respectively.

Table 2 shows the full experimental design and corresponding data obtained. The highest actual and predicted protease specific activities were Run 4 (98.64 U/mg) and Run 6 (100.13 U/mg), respectively, under predetermined factors (Run 4- Pressure: 23 MPa, Temperature: 42 °C; Run 6- Pressure: 23.83 MPa, Temperature: 41 °C), whereby the actual protease specific activity showed a slight difference from the predicted value. Meanwhile, the lowest actual and predicted protease specific activity also portrayed slight differences in Run 3 (26.11 U/mg) and Run 8 (28.90 U/mg), respectively, under the specific condition of variable factors (Run 3- Pressure: 19 MPa, Temperature: 42 °C; Run 8- Pressure: 21 MPa, Temperature: 42.41 °C). Moreover, the lowest actual and predicted fat residue in the defatted sample as shown in Table 2 was Run 1 (57.71% and 57.08%, respectively, at 19MPa and 40 °C).

Table 2. Factors and comparison between actual (Y) and predicted (FITS) responses.

Run No.	Factors		Responses			
			Specific Activity (U/mg)		Fat Residue (%)	
	X_1	X_2	Y_1	FITS 1	Y_2	FITS 2
1	19.00	40.00	93.28	90.61	57.71	57.08
2	23.00	40.00	61.54	46.71	60.36	61.51
3	19.00	42.00	26.11	30.20	75.58	75.15
4	23.00	42.00	98.64	90.57	59.91	61.26
5	18.17	41.00	91.71	88.48	65.75	66.65
6	23.83	41.00	86.16	100.13	61.58	59.96
7	21.00	39.59	30.45	40.60	58.13	57.91
8	21.00	42.41	28.31	28.90	71.01	70.51
9	21.00	41.00	47.94	41.45	57.14	60.02
10	21.00	41.00	33.28	41.45	58.75	60.02
11	21.00	41.00	31.99	41.45	60.36	60.02
12	21.00	41.00	48.26	41.45	61.93	60.02
13	21.00	41.00	45.80	41.45	61.93	60.02

X_1- Pressure (MPa), X_2- Temperature (°C), Y_1- Actual Specific Activity (U/mg), Y_2- Actual Fat Residue (%), FITS 1- Predicted Specific Activity (U/mg), FITS 2- Predicted Fat Residue (%).

Response surface regression for the optimization of the Sc-CO_2 process

A regression analysis was carried out to fit the mathematical models to the experimental data aiming at the optimal region for the response studied. By applying the multiple regression analysis, the empirical relationship between the independent variables and response variable can be expressed in the following quadratic, second-order polynomial equation (Eqn. 5 and 6) in terms of the coded values:

$$Y_1 = 41.45 + 5.82X_1 - 5.85X_2 + 52.85X_1^2 - 6.71\,X_2^2 + 52.1\,X_1X_2 \qquad \textbf{Eqn. 5}$$

$$Y_2 = 60.022 - 3.344X_1 + 6.299\,X_2 + 3.28\,X_1^2 + 4.18\,X_2^2 - 9.16X_1X_2 \qquad \textbf{Eqn. 6}$$

where Y_1 is the protease specific activity (U/mg); Y_2 is the fat residue (%); X_1 is the pressure (MPa) and X_2 is the temperature (°C).

From Eqn. 5, it was observed that the linear terms for pressure had positive effects whereas temperature had a negative effect on protease specific activity (Y_1). A similar trend was also found in the quadratic terms X_1^2 and X_2^2 on response Y_1. As for the interaction terms, X_1*X_2 had positive effects on the response Y_1.

On the other hand, Eqn. 6 demonstrated that the linear terms for temperature had positive effects, but the pressure had negative effects on the fat residue (Y_2) of the defatted sample. In quadratic terms, both X_1^2 and X_2^2 had positive effects on Y_2. Unlike Eqn. 5, the interaction terms X_1*X_2 had negative effects on the response Y_2 in Eqn 6.

Analysis of Variance (ANOVA)

Analysis of variance (ANOVA) was adopted in this study to examine the suitability and fitness of the experimental variables on the linear, quadratic and interaction terms (Sahimi and Ismail, 2021). Furthermore, it was conducted to analyse the statistical significance of the regression equation (Eqn. 5 and 6). Indication of a better fit of the RSM model to the experimental data is by referring to a larger F-value as it forecasts the quality of the whole model with regards towards all the design factors all at the same time (Bisht *et al.,* 2013). Apart from the F-value, the P-value is also important as it reflects the probability of the factors having very little or insignificant effect on the response. A low P-value ($p<0.05$) suggests the model to be statistically significant.

According to Yuan *et al.* (2017), the P-value can be categorized into 3 groups: i) $p<0.01$= "highly significant", ii) $0.01<p<0.05$ = "significant", iii) $p> 0.05$ = "not significant".

ANOVA analysis as shown in Table 3 shows that interaction and quadratic (square) factor had a significant effect on the model for the response variable of protease specific activity as indicated by higher F-value of 21.59 and 20.59, respectively, with low P-value of 0.001 and 0.002, respectively, compared to regression factor that is least significant with F-value of 12.99 and P-value of 0.002. Meanwhile, it is evident that there was no significant influence observed on protease specific activity given the F-value is small (1.08) with a high P-value of 0.389 ($p>0.05$) due to the linear effect of the variables as depicted in Eqn. 5. On the other hand, the ANOVA results (Table 3) demonstrated that the model was found to be significant for the response variable of fat residue in the defatted sample. The linear factor had the most significant effect on fat residue by obtaining the highest F-value (28.72) with a highly significant P-value (0.000).

The coefficient of determination (R^2) was calculated to determine the correlation measures for testing the goodness of fit of the second-order polynomial model. R^2 should be at least 0.80 for a model to be considered a good fit (Zaibunnisa *et al.,* 2009). The closer the R^2 to 1.0, the stronger the model and the better the correlation between the experimental and predicted values by the second-order polynomial model (Panwal *et al.,* 2011). The high R^2 value for the model response of protease specific activity and fat residue (90.27% and 93.03%, respectively) implies a high degree of correlation between the experimental and predicted values. The R^2 value in this study suggests that 90.27% or 93.03% for protease specific activity and fat residue, respectively, in the defatted sample was attributed to the independent variables, and only 9.73% and 6.97% of the total variation was not explained by the model. Both R^2 values being closer to 1.0 indicated that the models represent a better correlation between experimental and predicted values. According to Kadiri and Anand (2016), a smaller value of the adjusted R^2 than the R^2 value occurs when there are many terms present in the model which is insignificant and also, the sample size involved is not very large. However, in this study, the adjusted R^2 for protease specific activity and fat residue (83.32% and 88.05%, respectively) were noticeably close to the respective R^2 values (Table 3) which were 90.27% and 93.03%, respectively. This small difference between the R^2 and adjusted R^2 signifies that the models are adequate for the data (Patel *et al.,* 2011). Furthermore, the high values of R^2 and adjusted R^2 show the occurrence of high dependence and correlation between the experimental and predicted values of the response variables (Noor Eliza *et al.,* 2021).

Table 3. ANOVA of multiple regression models for the response variables

Source	Degree of freedom	Adjusted sum of square	Adjusted mean square	F-Value	P-Value	Status
Percentage of protease specific activity (R^2 = 90.27%; Adjusted R^2= 83.32%)						
Regression	5	8175.31	1635.06	12.99	0.002	Significant
Linear	2	272.56	136.28	1.08	0.389	Not Significant
Square	2	5184.69	2592.35	20.59	0.001	Significant
2-Way Interaction	1	2718.06	2718.06	21.59	0.002	Significant
Error	7	881.16	125.88			
Lack-of-Fit	3	617.62	205.87	3.12	0.150	Not Significant
Pure Error	4	263.54	65.89			
Total	12	9056.47				
Percentage of fat residue (R^2 = 93.03%; Adjusted R^2= 88.05%)						
Regression	5	331.039	66.208	18.69	0.001	Significant
Linear	2	203.465	101.733	28.72	0.000	Significant
Square	2	43.668	21.834	6.16	0.029	Significant
2-Way Interaction	1	83.906	83.906	23.68	0.002	Significant
Error	7	24.798	3.543			
Lack-of-Fit	3	7.479	2.493	0.58	0.661	Not Significant
Pure Error	4	17.319	4.330			
Total	12	355.837				

The lack-of-fit test is an important aspect in examining the suggested model fit with the reliable data obtained (Siti Roha *et al.,* 2022). For a model to be good and fit the data well, the lack-of-fit test must be insignificant (Patel *et al.,* 2011). If vice versa, the model should be rejected because of the noise or the model itself fails to represent the data in the experimental domain at points that are not included in the regression (Myers *et al.,* 2009; Siti Roha *et al.,* 2022; Srivastava *et al.,* 2021). The findings of this study (Table 3) revealed that the model predicted all the response variables (Y_1 and Y_2) were satisfactory and fit well with the experimental values as indicated by the non-significant lack-of-fit ($p > 0.05$) with P-value 0.150 and 0.661, respectively. Overall, the predicted model was statistically valid as recommended by the high goodness of fit and hence, the feasibility of the polynomial equation was established in Eqn. 5 and 6.

The optimum conditions of pressure and temperature of Sc-CO_2 based on protease specific activity and fat residue in the defatted sample are shown in Table 4. The feasibility of the experiments for target, maximum and minimum goal can also be seen on overlaid contour plots as depicted in Fig. 2. In overlaid contour plot, the white region is called the feasible region, an area such that the acceptable values for each response are between their respective contours (Khor *et al.,* 2016). Within the feasible region, the possible combination of parameter settings can be acquired (Khor *et al.,* 2016).

Based on Table 4 and Fig. 2, it was found that the optimum conditions for the two goals (target and maximum) were feasible to be carried out except for the minimum goal. Target and maximum goals were situated in the white area or within the feasible region whereas the minimum goal was situated in the grey area or non-feasible region. Only optimum conditions in target and maximum goals (X_1: 18.17 MPa and X_2: 41.33 °C) were further selected. This is because the responses obtained in both target and maximum goals (Y_1: 74.65 U/mg and Y_2: 70.46 %) meet the objective of the study wherein producing defatted sample with high protease specific activity at low fat residue under the optimum Sc-CO_2 conditions compared to the response in minimum goal (Y_1: 68.89 U/mg and Y_2: 56.91 %).

Table 4. Comparison values of target and predicted responses for different optimum conditions and experiment feasibilities

Goal		**Lower**	**Target**	**Upper**	**Optimum Condition**		**Predicted Responses (FITS)**		**F/NF**
					X_1	**X_2**	**Y_1**	**Y_2**	
Target	SA	26.11	98.63	98.64	18.17	41.33	74.65	70.46	F
	FITS 1	28.897	100.13	100.13					
	FR	57.14	75.57	75.58					
	FITS 2	57.08	75.15	75.15					
Maximum	SA	26.11	98.64	98.64	18.17	41.33	74.65	70.46	F
	FITS 1	28.90	100.13	100.13					
	FR	57.14	75.58	75.58					
	FITS 2	57.08	75.15	75.15					
Minimum	SA	26.11	26.11	98.64	19.66	40.00	68.89	56.91	NF
	FITS 1	28.90	28.90	100.13					
	FR	57.14	57.14	75.58					
	FITS 2	57.08	57.08	75.15					

SA- Specific Activity (U/mg), FR- Fat Residue (%), FITS 1- Predicted Specific Activity (U/mg), FITS 2- Predicted Fat Residue (%), X_1- Pressure (MPa), X_2- Temperature (°C), Y_1- Actual Specific Activity (U/mg), Y_2- Actual Fat Residue (%), F-Feasible, NF- Not Feasible

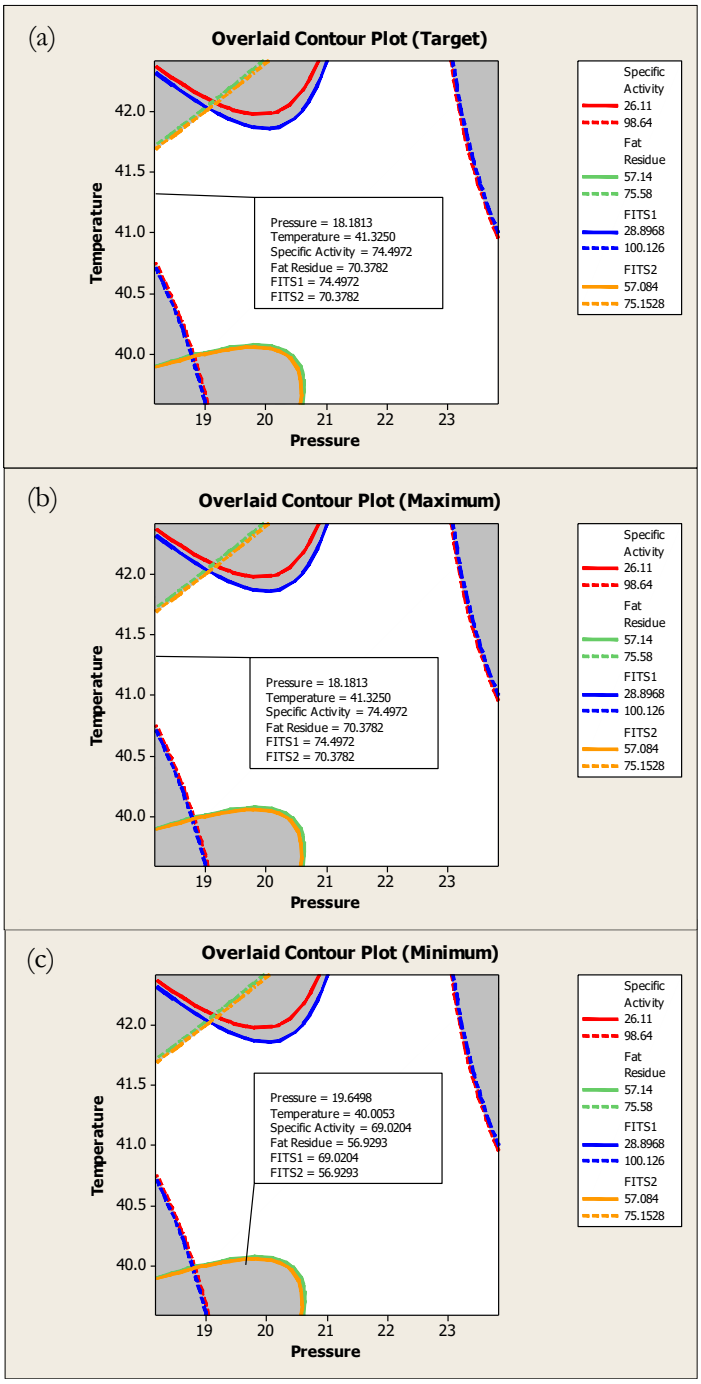

Fig. 2. Overlaid contour plot at the optimum condition for the (a) target, (b) maximum and (c) minimum goal.

Response surface and contour plot of the optimum condition

According to Xu *et al.* (2008), a 3D surface plot is the best way to understand and visualize the effect of independent variables on the response variables. Moreover, the shapes of the 2D contour plot either circular or elliptical detect whether the interaction between the variables is significant or not (Zainal *et al.,* 2013). The circular shape of the contour plot shows negligible or insignificant interactions between the corresponding factors whereas the elliptical or saddle shape of the contour plot shows significant interactions between the

corresponding factors (Rengadurai *et al.,* 2012) and represents some deep interaction between the independent variables (Nourani *et al.,* 2016). By using both the response surface plot and contour plot of each pair of independent factors, the optimal value of them and their interaction can be easily understood (Nourani *et al.,* 2016). Results of the present study showed that the contour plot was elliptical, hence, suggesting a significant interaction effect between pressure and temperature of Sc-CO_2 on protease specific activity and fat residue in the defatted sample (Fig. 3b and 3d).

The pressure and temperature in this study were conducted at milder conditions during the defatting process via Sc-CO_2 by taking into consideration the delicate nature of the enzymes and protein in the viscera sample. Vaquero *et al.* (2006) and Chun *et al.* (2011) also suggest that lipids can be selectively extracted without affecting the protein under mild Sc-CO_2 conditions. Fig. 3a-b shows the 3D surface plot and 2D contour plot for the influence of pressure and temperature on protease specific activity. It can be observed that the protease specific activity increased at two conditions: i) either at a lower level of pressure and temperature (<19 to <21 MPa; <40 °C to 42 °C), or, ii) at a higher level of pressure and temperature (>23 MPa; 40 °C to >42 °C). Hence, the variables (pressure and temperature) are dependent on each other on protease specific activity. Interaction between operating pressure and temperature does have a positive impact on the protease specific activity.

On the contrary, a combination of middle pressure and temperature in this study (Fig. 3a-b) produces the defatted sample with low protease specific activity. Low or loss of enzyme activities was due to the formation of covalent complexes called carbamates with free amino groups on the enzyme's surface as a result of the interaction between CO_2 with the enzyme (Ali-Nehari *et al.,* 2012; Uddin *et al.,* 2009). Furthermore, the enzyme activity may be partially inhibited by the carbamates because carbamates cause charge removal at lysine residues (Habulin and Knez, 2001; Kamat *et al.,* 1992, 1995; Park *et al.,* 2008). Based on the previous works, protease specific activity after the sample defatted via Sc-CO_2 which was conducted at 25 MPa and 45 °C were 10 and 19 U/mg in mackerel viscera (Asaduzzaman and Chun, 2015), 0.062 U/mg in squid viscera (Uddin *et al.,* 2009) and 0.05 U/mg in krill (Ali-Nehari *et al.,* 2012). The specific activity of protease obtained from this study is reasonably high which is 77.506 U/mg under optimum conditions of Sc-CO_2.

For higher efficiency of enzyme isolation, it is crucial to remove the lipid layer from the sample. To do so, more fish oil needs to be excluded from the viscera during the Sc-CO_2 extraction process. In Fig. 3c-d, the low fat residue in the defatted sample was successfully achieved after the Sc-CO_2 extraction process at low temperature (<40.5 °C) and low to moderate pressure (<22 MPa) of Sc-CO_2. Removing the lipid layer in the viscera proved that high protease specific activity can be achieved during protease extraction and purification of the defatted sample. The physical properties of Sc-CO_2 are directly influenced by the operating pressure and temperature where those parameters have a direct impact on the vapour pressure of the solutes as well as the density and viscosity of the supercritical fluid (Kuvendziev *et al.,* 2018; Lisichkov *et al.,* 2014).

The effect of pressure on oil yield is due to an increase in CO_2 density increases and hence, increased lipid solubility in Sc-CO_2 (Temelli, 2009). Hawthorne *et al.* (1992) also believed that the solubility of the oil in Sc-CO_2 is enhanced by elevating the pressure at a given temperature, thus, improving the total oil yield obtained. Similarly, Sarker *et al.* (2012) noticed that the changes in pressure (10- 40 MPa) had a significant effect on oil yield rather than the temperature (35 to 80 °C). At relatively high pressure (28 MPa), they observed the total oil yield extract (59.8–64.4%) increased with medium to high temperature (55-65 °C). Conversely, the oil yield started to gradually decrease with pressure when the temperature reached above 65 °C because of the reduction of CO_2 density and solvation power of the Sc-CO_2 (Sarker *et al.,* 2012). According to Najafi *et al.* (2022), the solubility of solute increases with the increase in temperature (40-60 °C) at a pressure of 10- 30 MPa due to the increment of the solute vapour pressure effect. They also deduced that the supercritical fluid diffusivity is enhanced when the vapour pressure of the extract is elevated with increasing temperature. In fact, the increase in pressure leads to an increase solvent power of CO_2 in particular, hence, the solubility of the solutes increases (Najafi *et al.,* 2022). Nonetheless, the impact of pressure can also be explained by the increase in solvent power and by strengthening intermolecular physical interactions between the solvent and solute (Jamalluddin *et al.,*

2022). Therefore, in relation of this behaviour with this study, more fish oil was excluded from the viscera (Fig. 1, No. 18) and left only a low amount of fat residue in the sample (Fig. 1, No. 17).

The majority of the study related to defatted samples via Sc-CO_2 for enzyme recovery only mentioned oil yield obtained after the Sc-CO_2 extraction process rather than fat residue left in the defatted sample. For instance, Asaduzzaman and Chun (2015) obtained the highest oil yield (20%) from mackerel muscle when conducting the Sc-CO_2 for 2 h at 45 °C and 25 Mpa. Similarly, Uddin *et al.* (2009) also reported the highest oil extracted from squid viscera (30%) after the Sc-CO_2 process at 45 °C, 25 Mpa for a duration of 2.5 h. Meanwhile, Park *et al.* (2008) found that at 35 °C and 25 Mpa, the oil yield obtained from mackerel viscera was low, but increased from 13 to 16% when temperature increased up to 45 °C. As for Lisichkov *et al.* (2014), they proclaimed that the increase of pressure (30 to 35 Mpa) would increase the yield of fish oil (2.70 to 34.13%) due to the increase of the CO_2 density at 40 °C, with a constant CO_2 flow rate of 3 g/min.

On the other hand, it was observed that the fat residue is high or not fully removed from the viscera after Sc-CO_2 at low pressure (< 19 MPa) when temperature above 42 °C (Fig. 3 c-d). This phenomenon was due to the reduction of CO_2 density and solvation power of the Sc-CO_2 as mentioned by Sarker *et al.* (2012). Besides, Lisichkov *et al.* (2014) and Kuvendziev *et al.* (2018) also observed a similar trend (decrease of total oil yield) when the temperature increased at lower pressure (20-30 MPa) causing a negative influence on the mass transfer. Sarker *et al.* (2012) postulated that the effect of temperature on the yield of fish oil was not well elucidated in the Sc-CO_2 extraction process probably due to more competing parameters involved in Sc-CO_2 (Jamalluddin *et al.,* 2022). Thus, Sarker *et al.* (2012) believed as they discovered the effect of pressure changes (10-40 MPa) on oil yield was more noticeable and had a significant effect rather than temperature (35- 80 °C).

By analysing the surface and contour plot, the predicted protease specific activity and the fat residue are observed to be 74.65 U/mg and 70.46%, respectively, which lies in the following ranges of the examined variables such as 18.2 MPa and 42 °C.

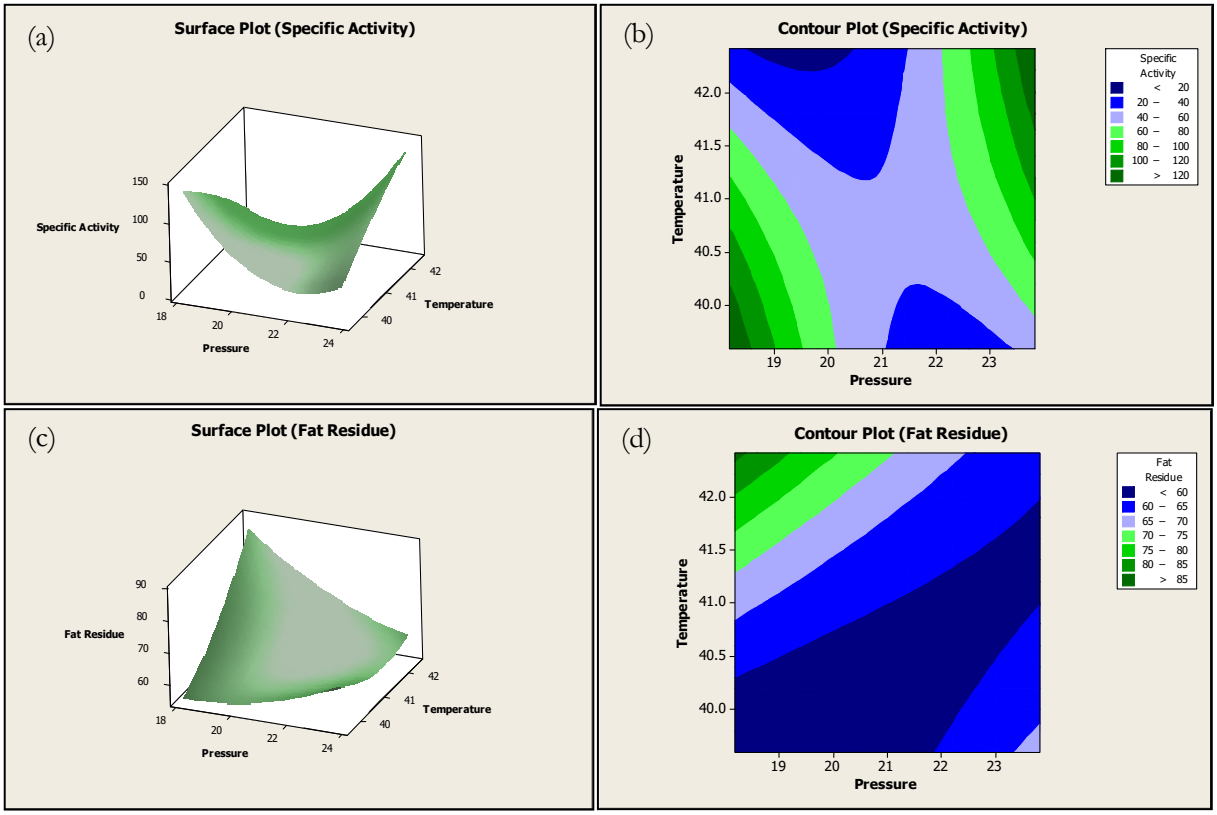

Fig. 3. Response surface and contour plot of protease specific activity (a, b) and fat residue (c, d) in defatted visceral of hybrid catfish after Sc-CO_2 extraction process at feasible optimum condition.

Verification of the Sc-CO_2 optimum condition on protease specific activity and fat residue in defatted sample

Verification of the feasible Sc-CO_2 optimum conditions (X_1= 18.2 MPa, X_2=42 °C) was performed and the result is shown in Table 5. The verified value of protease specific activity and fat residue of the defatted sample were identified with 77.51±0.06 U/mg and 71.17±0.38 %, respectively, which is close to their predicted value (74.65 U/mg and 70.46 %, respectively). The difference between the verification and predicted values for both responses was less than 5%. Therefore, it can be deduced that these models were sufficient to predict the response variables as well as confirm the validity and adequacy of the final reduced model fitted by RSM. Also, the model was significant and can be used to produce the defatted sample via Sc-CO_2 under optimum conditions.

Table 5. Comparison of the verified and predicted values of protease specific activity and fat residue at feasible optimum conditions.

Optimum condition	Responses	Verification values	Predicted values	Differences (<5%)
X_1: 18.2, X_2: 42	Protease Specific Activity (U/mg)	77.51 ± 0.06	74.65	2.86
	Fat Content (%)	71.17 ± 0.38	70.46	0.24

Where: X_1= Pressure (MPa), X_2= Temperature (°C), V= verification value, P= predicted value

CONCLUSION

In conclusion, the CCD was successfully employed in RSM to optimize the Sc-CO_2 conditions such as pressure and temperature for the response variables of protease specific activity and fat residue in defatted viscera. The optimum Sc-CO_2 conditions were found to be at a pressure of 18.17 MPa and a temperature of 41.33 °C. At these optimum conditions, high protease specific activity (77.51 U/mg) with low fat residue (71.17 %) was obtained. There were significant interactions between the pressure and temperature of Sc-CO_2 on protease specific activity and fat residue in the defatted sample as depicted in the elliptical shape of the overlaid contour plot. In the verification of the Sc-CO_2 optimum condition, the differences were less than 5% between the verified and predicted values for all the response variables. Hence, removing the lipid layer in the catfish viscera via the Sc-CO_2 process proved to be a feasible technique and acceptable in getting high protease specific activity as predicted by the RSM in MINITAB statistical software version 16. It is by removing finely dispersed lipids in the sample ease the subsequent isolation and purification of protease. The ability of the Sc-CO_2 to defat the sample with minimal denaturing proteins and enzymes in the defatted sample highlights the usefulness of this technology in substituting other organic solvents defatting process. Therefore, it is worthwhile to defat the sample via Sc-CO_2 before attempting the recovery of protease at optimum conditions.

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