Optimization of enzymatic hydrolysis conditions of Caspian kutum (*Rutilusfrisiikutum*) by-product for production of bioactive Peptides with antioxidative properties

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Abstract

The enzymatic hydrolysis was performed by Alcalase to recover the fish protein hydrolysate from Caspian kutum by-product (CB). The degree of hydrolysis (DH) was applied for monitoring the hydrolysis reaction of CB. The response surface methodology (RSM) was applied based on a D-optimal design to perform the optimization process for obtaining the high yield of CB protein hydrolysate. The effect of four independent variables including pH (7.5-8.5), temperature (45-55 °C), time (1-3 h), and enzyme concentration (0.5-1.5% w/w) on DH was studied. The results indicated that the predicted and actual values of the optimum condition had no significant difference. The optimum enzymatic hydrolysis conditions were achieved at pH 8.5, temperature of 55 °C, enzyme concentration of 1.5% w/w, and time of 3

h, which resulted in the maximum value of DH (19.08%). Antioxidant assays including DPPH scavenging and metal chelating activities showed that Caspian kutum protein hydrolysates (CKPH) had antioxidant properties.

Keywords: Fish Protein Hydrolysate, by-product, Optimization, Alcalase, hydrolysis, antioxidant.

1. Introduction

Caspian kutum (*Rutilus frisii kutum*) is the most common and cultured freshwater fish in northern Iran. It appears to be the most important fish of the Caspian Sea having the advantage of high nutritional value, good meat quality, and desirable taste. The Caspian kutum fish is a member of the family Cyprinidae from brackish water habitats of the Caspian Sea and from its freshwater tributaries. It is typically a medium-sized fish, reaching 45–55 cm in length, rarely 70 cm, and weighing up to 4.00 kg, rarely 5.00 kg. The rapid growth of fish consumption over the last years has led to increased amount of fish waste following their handling and processing including viscera, heads, cut-offs, bone, skin, fins, roes, and frames (*Iranian statistical yearbook 2015-2016*) that consist 75% of the total weight of the harvested fish (Rustad et al. 2011). These wastes are ordinarily discarded leading to increased food waste and disposal issues. Various researches have been performed to recycle valuable compounds such as proteins, polyunsaturated omega-3 fatty acids, minerals, and vitamins that can be a natural source for fulfilling the human micronutrients requirements (Noman et al. 2018; Zheng et al. 2018).

Several methods have been employed to extract valuable compounds using chemical and biochemical processes or their combination. Fish protein hydrolysate (FPH) is one of the best sources of bioactive peptides with miscellaneous activities, namely antioxidant, anticancer, and angiotensin-converting-enzyme (ACE) preventive (Bougatef et al. 2010). It was reported in previous researches that bioactive peptides are recognized as potential substitutes for some medicines because of their specific properties such as, tumor piercing capability, low toxicity profiles, and small size (Barras and Widmann 2011). Therefore, enzymatic hydrolysis method using protease has been extensively utilized to extract and isolate the bioactive peptides from fish by-products. Therefore, to control this procedure, type of enzyme is a key point since

different enzymes have different capabilities. Particularly, Alcalase has been found as a useful enzyme due to its great capacity to produce FPH (Aspmo et al.2005; Ovissipour et al. 2009; Roslan et al. 2015). Generally, Alcalase is capable of hydrolyzing fish protein at temperatures ranging from 50-70 °C and pH values ranging from 6-10 (Adler-Nissen 1986). Several studies indicated different ways for optimizing hydrolysis conditions. The functional properties or bioactivities of peptides after enzymatic hydrolysis depended on the type of proteases and experimental conditions during hydrolysis (Mendis et al. 2005; Nazeer and Anila Kulandai 2012; Ishak and Sarbon 2018; Arvanitoyannis and Kassaveti 2008). However, a wide range of the enzyme activity conditions is required for determining the optimum condition for each process parameter and achieving a good outcome (Roslan et al. 2015; Benjakul and Morrissey 1997; Rodrigues et al. 2009; Sami et al., 2013). Optimization should be conducted to find the optimum hydrolysis conditions, such as DH. In this regard, optimization by response surface methodology (RSM) has been widely used in the studies of fish hydrolysate products (Ovissipour et al. 2009; Thuy et al. 2014; Wasswa et al. 2008; Je et al. 2008).

Functional properties of proteins can be modified by hydrolysis using several chemical and enzymatic methods. Enzymatic hydrolysis due to its milder condition and selectivity is more preferred. The products of enzymatic hydrolysis of protein are proteoses, peptone, peptides, and free amino acids (Liu et al. 2010; Kang et al. 2018; Kristinsson and Rasco 2000). These products have been reported to exhibit antioxidant properties such as DPPH scavenging, metal chelating and reducing power of free radicals (Chalamaiah et al. 2013; Molla and Hovannisyan 2011; Naqash and Nazeer 2013; Nazeer et al., 2011). There are several publications on hydrolyzing fish proteins and evaluating the bioactivity and functional properties of resulted peptides. However, there is no report regarding hydrolysis of Caspian kutum fish by products. Therefore, the purpose of this study was to optimize the enzymatic

hydrolysis conditions of Caspian kutum by-products using RSM. In addition, antioxidant properties of Caspian kutum protein hydrolysates (CKPHs) were evaluated through different assays.

2. Materials and Methods:

2.1. Materials

Fresh Caspian kutum were purchased from a local market in Sari, Mazandaran Province, Iran, and transported to the laboratory in iceboxes. After washing, the fish were decapitated, evacuated, filleted and de-skinned, and the by-products were separated (i.e., viscera, tail, backbones, and fines). Afterwards, the resultant CB was ground twice using an industrial mixer (Jaltajhiz, Tehran, Iran) at a medium speed with a blade size of 5 mm. Next, they were packed in polyethylene bags and then frozen and kept at -20 °C until further analysis. Alcalase, a bacterial endoproteinase enzyme produced by a strain of *Bacillus licheniformis* with a proteolytic activity of 2.4 AU/mL (AU–Anson unit) and a range of the activity temperature between 35 and 70 °C (Novozymes, 2007), was purchased from the Novozymes (Tehran, Iran) and kept at 4 °C until the assays.

2.2. Proximate analysis

Proximate composition (moisture, ash, lipid and protein) was determined. Moisture content was determined by placing approximately 2 gr of sample into a pre-weighted aluminum dish (AOAC, 1991). Samples were then dried in an oven at 105°C until a constant mass was obtained. Ash content was estimated by heating the pre-dried sample in a crucible at 600°C until a white ash was formed (AOAC 1991). The total crude protein (N×6.25) in raw material was determined gravimetrically using Bligh and Dyer (1959) method (Bligh and Dyer 1959).

2.3.Preparation of Fish Protein Hydrolysate

For each run, a 50 g sample was put into a 250 mL Erlenmeyer flask and cooked in 85 °C water bath for 20 min to inactivate the endogenous enzymes according to (Guerard et al. 2002) method. Thereafter, the cooked materials were mixed with sodium phosphate buffer at 1:2 (w:v) ratio and homogenized using a Moulinex blender (model LM238,600 W,1.5 L) for about 2 min. The pH of mixture was adjusted to 8.5 (the optimum activity of Alcalase) using 0.2 NaOH. Then, the enzyme was added according to the experimental runs (**Table 1**). All reactions were conducted in a shaking incubator (model GTSL20,20 L, Jal tajhiz, ,Tehran, Iran) at a constant agitation of 200 rpm. After each sampling, the reactions were exposed to heating at 95 °C for 15 min to inactivate the enzyme (Ovissipour et al. 2012). The hydrolysates were subsequently cooled on ice, followed by centrifugation (Hermle Labortechnik GmbH Z 206 A, Speed Range: 200 - 6000 rpm with 50 rpm increments, max. radius: 11 cm, Wehingen, Germany) at 8000 ×g at 10 °C for 20 min to gather the supernatant. Finally, the soluble phase was dried using a spray-dryer (model DSD-03, Dorsa Tech, Iran) with inlet and outlet air temperature of 170 °C and 80 °C, respectively. The freeze dried hydrolysates were then stored at -80°C pending further analysis.

2.4.Degree of hydrolysis

The DH was measured based on Hoyle and MerrlTt (1994) method. After complete hydrolysis, 20% TCA was added to terminate the reaction and then centrifuged to gather the 10% TCA soluble material from the supernatant. Equation (1) was used to estimate DH:

$$\%\,DH = (10\%\,TCA\ soluble\ N\ in\ the\ sample\ /\ total\ N\ in\ the\ sample) \times 100$$

Equation (1)

2.5.Experiment for optimization

The RSM based on a D-optimal Design was applied for optimizing the enzymatic hydrolysis conditions of CB using Alcalase enzyme. The influences of the four independent variables, including A (pH), B (temperature, ºC), C (enzyme concentration, %w/w), and D (Time, h), at three levels (-1, 0, 1) on the DH were investigated by D-optimal algorithm. The coded and real values of the experimental design are summarized in **Table 2**.

To select the range of each independent factor, the results of an initial study were used and the minimum and maximum levels were appointed considering the highest DH. The experimental values for the DH under the different combinations of the independent factors are shown in **Table 2**. The D-optimal designs consisted of 25 treatments, including 5 replicates of the central points.

2.6.Antioxidant properties

Peptides obtained from Caspian kutum fish by-products protein hydrolysates (CKPHs) using centrifuge (3000 rpm, 15 min) and their antioxidant properties were assessed. The supernatant was collected and antioxidant properties were measured at concentrations of 100, 200, 400, 500 and 600 $g.L^{-1}$.

2.7.Ferric reducing power assay

The reducing power of CKPHs was measured according to the method reported by Jeevitha et al. (2014) (Jeevitha et al. 2014). The amount of 1 mL of each CKPHs concentration was mixed with1 mL of 0.2 M phosphate buffer (pH 6.6) and 2 mL of 1% potassium cyanoferrate. Then, the mixture was incubated at 50°C for 20 min. After that, 2 mL of tricholoroacetic acid (10%) was added in order to stop the reaction. The amount of 2 mL of this mixture was added to 2 mL of distilled water and 0.4 mL of ferric chloride (0.1%) was added to it and left at room temperature for 10 min. Then, the absorbance was determined at 700 nm using a UV spectrophotometer.

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143 **2.8.DPPH radical scavenging activity**

144 The DPPH radical scavenging activity of CKPHs was measured using Bersuder et al.
145 (1998) method with some modifications (Bersuder et al. 1998). 1 mL of different
146 concentrations of CKPHs was added to 500 µl of DPPH (0.16 mM in 95% methanol). Then
147 the mixture was vortexed for 30 s and kept at darkness for 30 min. A control sample was
148 made without adding sample solution to the reagent. Absorbance was determined at 517 nm
149 using a UV spectrophotometer. DPPH scavenging activity was calculated according to the
150 Equation 2:

151 $$\text{DPPH scavenging activity} = \frac{A_{control}-A_{sample}}{A_{sample}}\times 100$$

152 Equation (2)

153 **2.9.Fe^{2+} chelating activity**

154 Metal chelating activity of CKPHs was determined based on a method developed by
155 Decker and Welch (1990) (Decker and Welch 1990). The amount of 1 mL of different
156 concentration of CKPHs was mixed with 3.7 mL distilled water and 0.1 mL of 2mM $FeCl_2$
157 and 0.2 mL of 5 mM of ferrozine. The mixture was vortexed for 30 s and kept at room
158 temperature for 10 min and the absorbance was measured at 560 nm using a UV
159 spectrophotometer. The metal chelating activity of CKPHs was calculated according to
160 Equation (2).

161 **3. Statistical analysis**

162 The RSM was statistically analyzed by the Design-expert software, Version 7.1.5
163 (Stat-ease Inc., Minneapolis, Minn., U.S.A.). Analysis of variance (ANOVA) was used to
164 determine the significance of model, coefficient estimation of each component at 95%
165 confidence level. The linear equation was the most fitted model to describe the effect of
166 independed variables on DH:

$$Y = \beta_0 + \sum \beta_0 Xj \quad \text{Equation (3)}$$

where, Y is the response (DH), β_0 is the intercept and j is independed variable.

4.Results

4.1. Proximate composition

Proximate composition of Caspian Kutum by-product is shown in **Table 3**. The composition of protein hydrolysates commonly was influenced by enzyme, pH, incubation time and analytical methods used for hydrolysis. In the present study, the Alcalase enzyme with suitable pH and temperature was applied to obtain the protein hydrolysate. The proximate analysis in term of protein, lipid, moisture, and ash contents of both the crude wet sample and freeze-dried protein hydrolysate of Caspian Kutum by-product were determined. The crude wet sample of CB by-product showed higher moisture content (78.95%) and least ash (2.17%) content. The protein hydrolysate of CB by-product showed protein content of around 87.16%, which was in agreement with earlier findings (Sheriff et al. 2013).

The RSM D-optimal design was applied for the optimization of hydrolysis condition (pH, temperature, enzyme concentration, and time) in CB. The linear equation obtained through the RSM is described below:

$$Y = -16.86 + 2.27\,A + 0.22\,B + 2.84\,C + 0.12\,D \quad \text{Equation (4)}$$

Where,Y, A, B, C, D are DH, pH, temperature, enzyme concentration, and time, respectively. Validation parameters of the selected model were evaluated by ANOVA. The results of linear model according to ANOVA are presented in **Table 4**. The ratios greater than 4 are indicative of adequate model discrimination (Bezerra et al. 2008). The F-value and P-value of model

were 60.30 and 0.0001, respectively, showing that the model was statistically significant (P<0.5). The lack of fit (LOF) refers to a measure of how well the model fits the data and compares the residual error with the pure error from the replicated design points. A model with a significant LOF demonstrates that the residual error is considerably larger than the pure error, and the model is not a good predictor of response (Paul Singh 1996) . As shown in **Table 4**, the P-value of the LOF was 0.67, suggesting that this result was not statistically significant and the selected linear model was a good predictor of DH. The adequate precision represents the signal-to-noise ratio (S/N) providing the measurement of the predicted response range relative to its associated error ratio (**Table 3**).

It is possible to visualize the effect of the independent variable on the DH through the three–dimensional response surface, which are based on the linear model (**Figure** 2a-d). **Figure** 1b exhibits the influence of the enzyme concentration and pH on the DH of CB. **Figure 1a** displays the three-dimensional plot for the DH as a function of the temperature and pH. It was found that the DH increased with elevations in the temperature and enzyme concentration. The highest DH was obtained at a temperature of 55 °C. It was revealed that as Ph increased, the DH increased too. **Figure 2c** displays the impacts of the enzyme concentration and temperature on the DH of CB. According to the results, increasing the enzyme concentration would elevate the DH value. The highest DH value was achieved at 1.5% w/w enzyme concentration. The reaction time did not show significant effect on DH (p<0.5) (**Table 4**).

After the evaluation of the validation parameters for the model, the optimization process was carried out to obtain ideal conditions for the highest level of the DH. Derringer's desirability function was utilized for the best optimum condition:

$$D = \sqrt[m]{d_1, d_2 \dots d_m} \qquad \text{Equation (5)}$$

In Equation 3, ‘m’ is the number of responses studied in the optimization process, and ‘d’ is the individual desirability function of each response. Derringer’s desirability function (D) can take values from 0 to 1. The value above 0.7 demonstrates the suitability of the selected optimum point for the process optimization (Granato and Ares 2014).

The result of optimization process is displayed in **Figure** 2.

The experimental validation of data is presented in **Table 5**. The observed and anticipated values were compared to assess the validity of the above model. These findings apparently confirm the validity of the model.

The results of antioxidant properties of CKPHs showed that increasing the protein hydrolysates led to an increase in the reducing power (**Figure 3**). Reducing power indicates the ability of an antioxidant to reduce the free radicals. It was found that the reducing power for CKPHs with concentrations of 100, 200, 400, 500 and 600 $g.L^{-1}$ were respectively, 0.35, 0.40, 0.48, 0.60, and 0.78. Moreover, DPPH scavenging activity of CKPHs increased significantly with increasing the concentration (**Figure 4**). The results of metal chelating effect are depicted in **Figure 5**. Increasing CKPHs from 100 to 600 $g.L^{-1}$ led to increased metal chelating effect from 18.20 to 61.33%. It can be concluded that antioxidant properties had a direct increasing relationship with concentration of CKPHs.

4. Discussion

The hydrolysis of CB by Alcalase was highly affected by the different experiment conditions, such as pH, temperature, enzyme concentration to obtain the superior yield of the CB protein hydrolysate.

The ANOVA results also demonstrated that the linear model term of pH (A) was significant ($P < 0.05$), followed by temperature (B) and enzyme concentration (C); nonetheless, time (D) was not statistically significant ($P > 0.05$). Equation 2 indicated that the main effect of pH,

242 temperature, enzyme concentration, and time had positive contributions to DH. Comparing
243 the coefficients of linear terms presented that concentration and pH with coefficients of
244 respectively 2.84 and 2.27 exerted a strong influence on the DH value. Meanwhile,
245 temperature with coefficient of 2.22 showed less effect (Equation 2).
246 In order to verify the optimum condition, the theoretical values of the optimum points were
247 tested actually by triplicate measurements and evaluated by paired T test analyses. If the
248 desirability value is close to 1.0, the offered conditions are appropriate to achieve the highest
249 DH. The model could predict the desirability value of 0.92.
250 The adequate precision value for the selected model was 26.41, showing that the signal-to-
251 noise ratio was very good. Further evaluation of the validation parameter was carried out by a
252 coefficient of determination value (R^2). The fitted model showed the experimental data with a
253 high coefficient of determination value (R^2 = 0.92). Also, the P-value of 0.8 (**Table 5**)
254 indicated the anticipated and actual results were not significantly different. These findings
255 verified the optimum conditions.
256 The 3D plot of the enzyme concentration confirmed that concentration was the major factor
257 affecting the DH. Moreover, the elevated concentration appeared to increase the DH.
258 Likewise, increasing pH was associated with an increase in the DH; however, this change
259 was less significant than that by concentration.
260 This demonstrated the effect of the temperature and pH on the enzyme activity (the enzyme
261 concentration and time were maintained at their mean levels). The findings indicated that the
262 operating variables, namely pH, temperature and enzyme concentration, affected the
263 hydrolysis with Alcalase. Similar results have been reported following the application of the
264 same commercial multifect-neutrase and Alcalase for the hydrolysis of the visceral waste
265 proteins of an Indian freshwater major carp (*Catlacatla*) (Bhaskar et al. 2008), enzymatic
266 hydrolysis of shortfin scad (Decapterus macrosoma) myofibrillar protein (Kang et al. 20018)

and, Shortfin scad (Decapterus Macrosoma) skin gelatin hydrolysate (Rasli and Sarbon 2018).

The optimum hydrolysis conditions were achieved at pH 8.5, temperature of 55 °C, and enzyme concentration of 1.5% w/w with the DH value of 19.08% after hydrolyzing for 180 min. The RSM and desirability function method appeared to be effective in determining the optimum condition for the highest level of the DH. In a previous research concerning the influence of enzyme concentration, time, temperature, and pH on DH of tuna fish viscera, it was shown that increasing Alcalase concentration from 1.0% to 1.5 %, temperature from 30°C to 40°C and incubation time from 60 min to 240 min significantly enhanced the DH value. Gueúrard et al. also reported a linear increasing relation between Alcalase concentration and DM of protein extract from yellowfin tuna wastes (Guérard et al. 2001). In another research, the effect of pH, temperature and enzyme to substrate ratio (E/S) on protein hydrolysis of dogfish muscles was investigated. They found that hydrolysis was optimized at pH of 8.3, the reaction temperature of 53.6°C and E/S of 3.6%. It was highlighted that increasing E/S upper than four could cause enzyme inhibition meaning that enzyme hydrolyse itself (Diniz and Martin 1996). Recently, Valencia et al. (2014) studied the effect of substrate, product and thermal inactivation using Alcalase assisted hydrolysis of salmon muscle protein. They found that the hydrolysate products are the key factor in reducing the reaction rate (Valencia et al. 2014). However, no inhibition effects were observed in the determined condition of Alcalase hydrolysis of Caspian kutum wastes.

Enzymatic hydrolysis has been used for proteins from various food sources to achieve the desired bioactivity and functional properties. Several reports have focused on producing bioactive peptides from fish proteins (Awuor et al. 2017; Cheung et al. 2012; Šližytė et al. 2009).

Protein hydrolysis allows producing several peptides that are capable of chelating pro-oxidants such as metals and/or reducing free radicals. In the current study, it was found that antioxidant activities including reducing power, DPPH scavenging and metal chelating ability were enhanced directly with increasing the CKPHs concentration. The reducing power shows the ability of an antioxidant to donate electron or hydrogen to free radicals in order to quench their pro-oxidant activity (Chalamaiah et al. 2015). There are few researches reporting a direct correlation between reducing power of fish bioactive peptides and their antioxidant activity (Bougatef et al. 2009; Bordbar et al. 2018). DPPH is a stable radical that gives a strong absorption band at 517 nm. When a DPPH solution changes in color, it indicates the presence of antioxidant compounds in the solution When DPPH radicals meet a proton-donating substrate like antioxidant, the radicals would be scavenged and the absorbance is decreased (Liu et al. 2010). The DPPH activity illustrated in Figure 4 revealed a dose depended relationship for CKPHs. Some factors such as substrate, protease type, hydrolysis condition, peptide composition, molecular size of the peptides, and sequence might influence the radical scavenging activity. A literature review demonstrated that antioxidant activity of protein hydrolysates was related to the DH since DH mainly influence the molecular weight and amino acid residue composition of protein hydrolysate and consequently antioxidant activity (Ramezanzade et al. 2018). Our results were in good agreement with previous researches (Chalamaiah et al. 2013; Intarasirisawat et al. 2012). in this regard Bordbar et al. (2018) conducted that the alcalase-generated proteolysates obtained after 8 h of proteolysis of stone fish flesh showed the most potent antioxidant activity in terms of DPPH• radical scavenging activity (Bordbar et al. 2018). It is believed that some divalent metals such as iron and copper can act as pro-oxidants by receiving electrons and producing free radicals. In this research, the significant increase of metal chelating activity was observed in higher CKPHs concentrations. Our results were in agreement with Sheriff et al. (2013) and (Hmidet et al.,

316 2011) reports that investigated the antioxidant activity of protein hydrolysates from
317 backbones of *Rastrelliger kanagurta* and cuttlefish (*Sepia officinalis*) muscles, respectively
318 (Hmidet et al. 2011). These results revealed the potential of peptide fractions recovered from
319 Caspian kutum wastes as source of natural antioxidants for use in food products and
320 pharmaceutical industry.
321
322 **5.**Conclusion
323 This study's analysis of Caspian Kutum by-product (CB) reported its crude protein, ash, lipid
324 and moisture. In the current study, it was found that increasing temperature, pH and Alcalase
325 concentration in the proteolysis of Caspian kutum wastes significantly ($P<0.05$) increased the
326 DH. However, time did not exert any significant effect ($P>0.05$). The optimum condition of
327 enzymatic reaction was determined as follows: temperature of 55°C, pH of 8.5 and Alcalase
328 concentration of 1.5%. Future investigations may focus on using higher concentrations of
329 enzyme in model systems in order to industrial scale up of the hydrolysis reaction. Reducing
330 power, DPPH scavenging and metal chelating activity assays revealed a direct correlation
331 between antioxidant ability and concentration of CKPHs and this valuable source could be
332 used in functional foods to alleviate high blood pressure, as well as as to increase products
333 shelf life. However, in vivo availibility, potency and safety must be determined before the
334 products can be used for thearapeutic purposes.
335
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Table 1. Independent variables and their coded and actual levels used in RSM.

Factor	Symbol	Coded Level		
		-1	0	1
pH	A	7.5	8	8.5
Temperature (°C)	B	45	50	55
Enzyme Concentration (%)	C	0.5	1	1.5
Time (h)	D	1	2	3

Table 2. The experimental design of RSM (actual values) and obtained values for the DH.

	Factor1	Factor 2	Factor 3	Factor4	Response
Run	A:pH	B:Temperature	C:Concentration	D:Time	DH
1	7.5	55	0.5	1	14.25
2	8.5	50	0.5	1	14.75
3	8	50	0.75	2	13.38
4	8	55	1	2	17.16
5	7.5	45	1	1	11.75
6	8.5	55	1.5	3	20.47

7	8.5	45	1.5	1	16.82
8	8	45	0.5	1	13.29
9	8.5	55	1	1	17.27
10	7.5	50	1	2	14.3
11	8.5	45	1	3	15.38
12	8	50	1.5	2	16.57
13	7.5	45	0.5	3	12.1
14	8.5	55	0.5	3	15.83
15	7.5	45	0.5	3	12.1
16	8.5	45	1.5	1	16.82
17	7.5	45	1.5	3	14.62
18	8.5	50	1	2	16.32
19	7.5	55	1	3	15.07
20	7.5	55	1.5	1	16.51
21	8	45	1	2	14.46
22	8.5	55	1.5	3	18.35
23	7.5	55	0.5	1	13.99
24	8.5	45	0.5	2	14.21
25	8.5	55	0.5	3	15.83

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558 **Table 3**. Proximate composition of *Caspian Kutum* by-product (CB).

Proximate composition (%)	**Raw material**	**FPH**
Crude protein	15.01	87.38

Ash	2.19	3.95
Lipid	4.73	1.61
Moisture	78.88	7.52

Table 4. Analysis of variance (ANOVA) for response surface linear model

Source	Sum of Squares	df	Mean Square	F-value	P-value (Prob> F)	
Model	92.33	4	23.08	60.30	< 0.0001	significant
A-pH	24.97	1	24.97	65.22	< 0.0001	
B-Temperature	23.93	1	23.93	62.50	< 0.0001	

C-Concentration	31.95	1	31.95	83.46	< 0.0001	
D-Time	0.24	1	0.24	0.62	0.44	
Residual	7.66	20	0.38			
Lack of Fit	5.38	15	0.36	0.78	0.67	not significant
Pure Error	2.28	5	0.46			
Cor Total	99.98	24				
$R^2 = 0.92$						
Adequate Precision = 26.41						

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579 **Table 5**. The predicted value of responses at optimized conditions

Theoritical value of DH	Experimental value of DH	P-value
19.10 ± 0.30	19.03 ± 0.20	0.8

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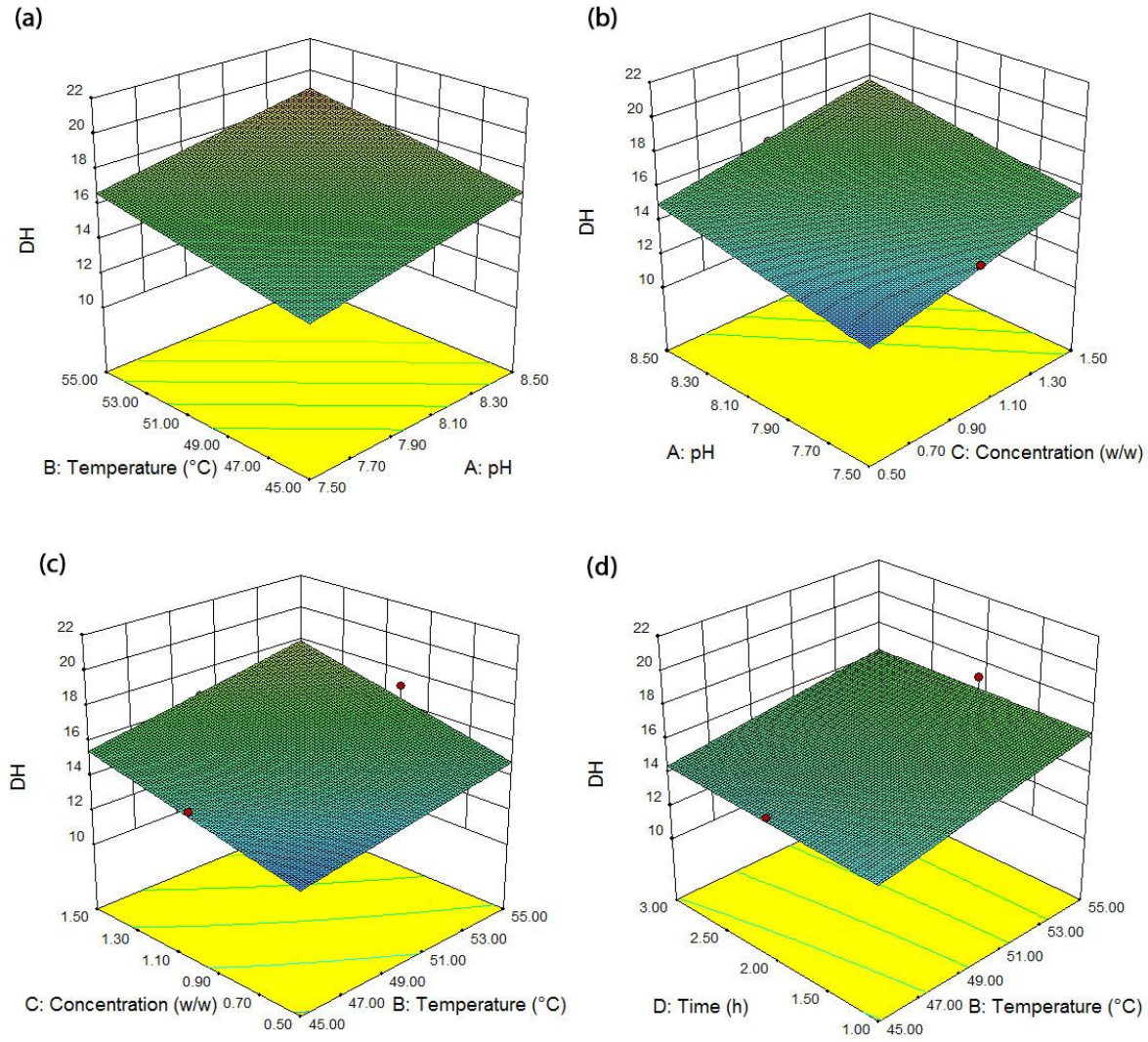
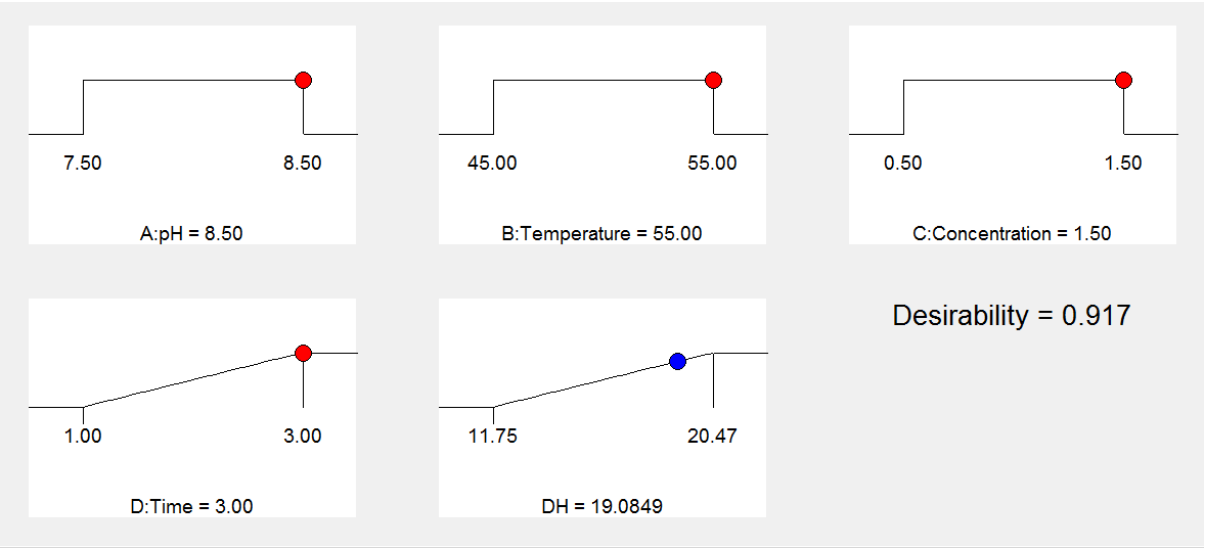


Figure 1: Response surface graph for the DH as a function of (a) pH and temperature, (b) pH and enzyme concentration, (c) temperature and enzyme concentration, (d) time and temperature



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592 **Figure 2**. Schematic representation of the optimum values of the factors, response, and their corresponding

593 levels

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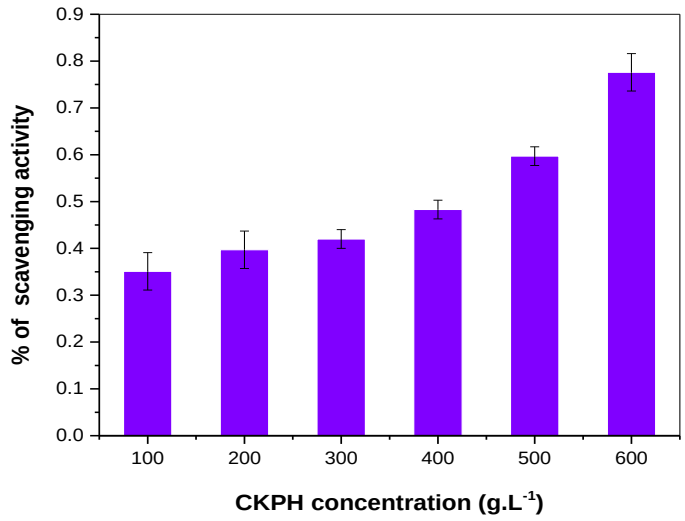

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598 **Figure 3**. Ferric reducing power of Caspian kutum protein hydrolysate

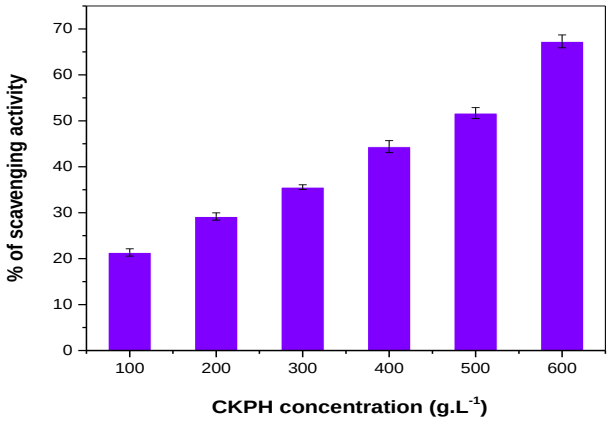
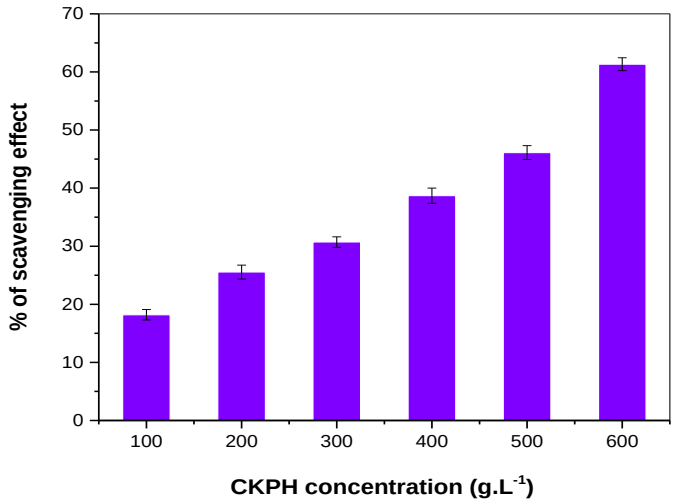


Figure 4. DPPH radical scavenging activity of Caspian kutum protein hydrolysate



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612 **Figure 5**. Metal chelating activity of Caspian kutum protein hydrolysate